3. MATERIALS AND METHODS

3.1 Study sites

The study sites were located at Makum coal fields of Tinsukia district of Assam, India approximately 65 Km away from Indo-Burma international border along with Assam-Arunachal Pradesh border (Fig. 7). Three chronosequence collieries viz. Ledo colliery (< 5 yrs), Tirap colliery (< 10 yrs), Tikak colliery (> 20 yrs) and one forest were selected for the present investigation (Plate 1 and 2). The region has subtropical and high humid climate. The geographical locations and climatological information of the sites are presented in Table 3 and Fig. 8 respectively.

Figure 7: Map showing the study sites (red dots) (prepared using ATLAS published map and GPS data). L=Ledo, TP=Tirap, TK=Tikak and F=Forest.
Photo plate 1: Sampling sites showing colliery and spoil soils (A-F). (A: Ledo colliery; B: Tirap colliery; C: Tikak Colliery; D: acid mine drainage; E-F: spoil soils).
Table 3: Geographical locations and altitude of the study sites.

<table>
<thead>
<tr>
<th>Study sites</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Altitude (asl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ledo colliery</td>
<td>27°17.272' N</td>
<td>95°45.012' E</td>
<td>665 ft</td>
</tr>
<tr>
<td>Tirap colliery</td>
<td>27°17.740' N</td>
<td>95°45.991' E</td>
<td>931 ft</td>
</tr>
<tr>
<td>Tikak colliery</td>
<td>27°16.748' N</td>
<td>95°44.371' E</td>
<td>600 ft</td>
</tr>
<tr>
<td>Forest</td>
<td>27°15.948' N</td>
<td>95°44.071' E</td>
<td>1750 ft</td>
</tr>
</tbody>
</table>

Maximum rainfall was recorded in the month of July during all the three years of the field work. While, minimum rainfall was recorded during winter (February) in 2010 and autumn (November) in 2011 and 2012. The year 2011, however, received less rainfall (1897.8 mm) as compared to the years 2010 (2779 mm) and 2012 (2576.9 mm). The monthly mean minimum temperatures ranged between 10.1-25.6 °C in
2010, 10.3 to 25.2 °C in 2011 and 10.7-25.5 °C in 2012. While, monthly mean maximum temperatures ranged between 25.1-32.4 °C in 2010, 22.5-33.3 °C in 2011 and 22.5-32.7 °C in 2012. The mean minimum temperature was recorded in the month of January, while, mean maximum temperature was recorded in the month of August. Monthly mean relative humidity ranged between 72-91 % in 2010, 70-88 % in 2011 and 74-90 % in 2012. Maximum relative humidity was recorded in the month of July.

3.2 **Collection of rhizospheric soil and root samples**

Soil samples were collected from all the four sites for a period of two years (2010-2012) during late summer (August) and late winter (February) seasons. Ten different plants were randomly selected in each study sites of a particular season for collection of soil and root samples. Rhizospheric soil (about 200 g fresh soil) and fine root samples of the selected plants were collected aseptically. Soil sampling was done using V-shaped technique (Bashan and Wolowelsky, 1987) from a depth of < 20 cm. Soil and root samples of a single plant were placed in individual plastic bags and transported to the laboratory. The individual samples collected were referred as 'sub-samples'. A portion of the soil sub-samples of same site and season were mixed in equal proportions to make composite sample. The samples were kept at 4 °C until they were processed.

3.3 **Analysis of soil physico-chemical characteristics**

3.3.1 **Temperature**

Soil temperature (°C) was measured by using a soil thermometer during soil sampling. To maintain uniform depth of measurement, pilot hole of 10 cm was drilled using nail of same diameter as that of the thermometer through wooden block (Fig. 9). Soil temperature was recorded after 2 minutes of insertion of the thermo-meter into the pilot hole.

3.3.2 **pH**

pH of the soil samples was determined using electronic digital pH meter (Eutech Cyberscan 510) in 1:5 (w/v) soil: water suspensions. Ten gram of air dried soil sample
was dissolved in 50 ml distilled water and the suspension was stirred with glass rod intermittently for 30 minutes. The suspension was kept standing for 1 hr in order to get stable reading. The pH was measured directly after calibration of the instrument with buffer solutions of pH 9.2 and 4.0.

3.3.3 Moisture content

Moisture content (%) of soil samples was determined according to the method as described in Methods Manual, Soil Testing in India, Department of Agriculture and Cooperation, Government of India (Anonymous, 2011). Ten gram fresh soil was dried in hot air oven at 105 ± 5°C until constant weight was observed. The formula used for calculating the soil moisture content (%) is as follows:

\[
\text{Soil moisture content (\%) } = \frac{W_2 - W_3}{W_3 - W_1} \times 100
\]

Here,

- \(W_1\) = Weight of foil (g)
- \(W_2\) = Weight of moist soil + foil (g)
- \(W_3\) = Weight of dried soil + foil (g)

3.3.4 Organic carbon

Titrimetric chromic acid wet oxidation method of Walkley and Black (1934) was followed for the determination of soil organic carbon (C\(_{\text{org}}\)). Organic carbon when treated with K\(_2\)Cr\(_2\)O\(_7\) and concentrated H\(_2\)SO\(_4\) gets oxidized. The un-oxidized organic carbon was titrated with 0.5N Fe (NH\(_4\))\(_2\)(SO\(_4\))\(_6\)H\(_2\)O (Mohr’s salt). The titre was inversely related to the amount of carbon present in the soil sample. Air-dried and sieved (0.2 mm) 0.5 g soil sample, 5 ml 1N K\(_2\)Cr\(_2\)O\(_7\) and 10 ml conc. H\(_2\)SO\(_4\) were taken in an oven dried 500 ml conical flask. They were mixed well and kept for 30 minutes. Thereafter, 100ml of distilled water, 5 ml 85% orthophosphoric acid and 5 ml 2% sodium fluoride were added to the mixture. Two/three drops of diphenylamine indicator were added and it was titrated with 0.5N Fe (NH\(_4\))\(_2\)(SO\(_4\))\(_6\)H\(_2\)O until colour changed to bright green through blue. Same procedure was followed for blank except soil was not used. The volume of 0.5N Fe (NH\(_4\))\(_2\)(SO\(_4\))\(_6\)H\(_2\)O used in blank (B ml) and sample (S ml) was noted. Three replicates were maintained in each case. The following formula was used to calculate the \(C_{\text{org}}\) present in the sample.
\[ C_{\text{org}}(\%) = \frac{\{0.5 \times (B - S) \times 0.003\}^{***} \times 1^* \times 1.33^{**} \times 100}{W} \]

Where

\( W \) = Weight of the soil  
\( B \) = Volume of 0.5N Fe (NH\(_4\))\(_2\)(SO\(_4\))\(_6\)H\(_2\)O used in blank titration  
\( S \) = Volume of 0.5N Fe (NH\(_4\))\(_2\)(SO\(_4\))\(_6\)H\(_2\)O used in sample titration  
* = Normality of K\(_2\)Cr\(_2\)O\(_7\)  
** = Correction factor due to 77% recovery by this method (100/77)  
*** = Amount of carbon in gram oxidized by K\(_2\)Cr\(_2\)O\(_7\)  

(1 ml of 1N K\(_2\)Cr\(_2\)O\(_7\) = 1 meq = 3 mg of organic carbon = 0.003 g of carbon)

The soil organic carbon <0.50% was interpreted as low, between 0.50-0.75% as medium and >0.75% as high.

### 3.3.5 Available nitrogen

Alkaline potassium permanganate method of Subbiah and Asija (1956) was followed to determine the amount of available nitrogen in soil when soil was distilled in Kjeldahl distillation flask with excess of alkaline KMNO\(_4\) solution, the nascent oxygen liberated oxidized the organic matter of the soil and ammonia was released. The released ammonia remains unstable in alkaline medium and was readily distilled and absorbed in known volume of H\(_2\)SO\(_4\). The excess of which was titrated with NaOH using methyl red as the indicator. Twenty gram air-dried and sieved (0.2 mm) soil was taken in distillation flask. Twenty ml of water and 100 ml of freshly prepared 0.32% KMNO\(_4\) solution were added to the distillation flask. The outlet of the condenser of the distillation assembly was dipped in conical flask containing 25 ml of N/50 H\(_2\)SO\(_4\) and 2-3 drops of methyl red indicator. The distillation flask was corked immediately after putting 100 ml of 2.5 % NaOH solution to it. The distilled ammonia gas from distillation flask was collected in H\(_2\)SO\(_4\) solution and was continued until evolution of ammonia ceased completely (tested by bringing a moist red litmus paper near the outlet of the condenser, which turned blue and remained so long as ammonia was being evolved). Excess of H\(_2\)SO\(_4\) was titrated against N/50 NaOH till the colour changed from pink to yellow. The volume of NaOH used was recorded. The following formula was used to calculate the available nitrogen present in the sample and was expressed as Kg/ha.
Available Nitrogen (Kg/ha) = \frac{(T - X) \times 0.00028 \times 10^6 \times 2.24}{W}

Where

\( W \) = Weight of the soil
\( T \) = Volume of N/50 H\textsubscript{2}SO\textsubscript{4} used
\( X \) = Volume of N/50 NaOH used in titration (titrate-value)
\( (T-X) \) = Volume of N/50 H\textsubscript{2}SO\textsubscript{4} used for NH\textsubscript{3} absorption

\( * \) = Amount of nitrogen in gram absorbed by 1 ml N/50 H\textsubscript{2}SO\textsubscript{4}

(1 ml N/50 H\textsubscript{2}SO\textsubscript{4}=0.02 meq. of N = 0.28 mg N = 0.00028 gm N)

The available nitrogen <250 Kg/ha was interpreted as low, between 272-544 Kg/ha as medium and >500 Kg/ha as high.

3.3.6 Available phosphorus

The available soil phosphorus (P) was determined by following Brays method (Bray and Kurtz, 1945). Five gram air-dried and sieved (2 mm) soil was taken in an oven dried 100 ml conical flask along with 50 ml of extracting solution (15 ml of 1N NH\textsubscript{4}F and 25 ml 0.5 N HCl to 460 ml distilled H\textsubscript{2}O). One tea spoon of ash was added to mixture. The mixture was shaken for 5 minutes and filtered through Whatman no. 42 filter paper. Five ml aliquot of the filtrate was taken into 25 ml volumetric flask. Thereafter, 5 ml extracting solution, 5 ml Dickman and Bray’s reagent and 1 ml stannous chloride solution (10 gm of SnCl\textsubscript{2} dissolved in 25 ml concentrated HCl) were added and the final volume was made up to 25 ml by adding distilled H\textsubscript{2}O. Absorbance was taken at 660 nm after 5 mins of incubation. Available P was determined by plotting the value in K\textsubscript{2}H\textsubscript{2}PO\textsubscript{4} standard curve. The following formula was used for calculation of available P\textsubscript{2}O\textsubscript{5} (Kg/ha) in the sample.

Available P\textsubscript{2}O\textsubscript{5}(Kg/ha) = A \times 50^* \times 2.24^{**} \times 2.29^{***}

Where

\( A \) = Concentration of P from standard curve
\( * \) = Total dilution
\( ** \) = Conversion of ppm to Kg/ha
\( *** \) = Conversion of available P to P\textsubscript{2}O\textsubscript{5} (Kg/ha)
3.3.7 Available potassium

The available soil potassium was extracted using 1 M neutral ammonium acetate and was determined by flame photometer method (Toth and Prince, 1949). A standard curve was prepared using flame photometer by atomizing 0 and 20 µg K/ml solution. These readings were plotted against the respective K contents. The points were connected with a straight line to obtain a standard curve. Five gram air-dried and sieved (0.2 mm) soil was taken in an oven dried conical flask and 25 ml of ammonium acetate extractant (NH4C2H3O2) was added to it. The mixture was shaken for five minutes and filtered. Available potassium in the filtrate was determined using flame photometer. The following formula was used for calculation of available potassium (K2O) in the soil sample.

\[
\text{Available K}_2\text{O (Kg/ha)} = C \times 5^* \times 2.24^{**} \times 1.20^{***}
\]

Where

- \(C\) = Concentration of K (ppm) as read from the standard curve.
- \(^*\) = Dilution factor
- \(^{**}\) = Conversion factor of ppm to Kg/ha
- \(^{***}\) = Conversion factor of available K to available K2O

3.3.8 Available calcium and magnesium

The available soil Ca and Mg were extracted using ammonium saturation method and concentration was determined by complexometric titration method of Schwarzenbach *et al.* (1946). Ten gram air-dried and sieved (0.2 mm) soil was saturated with 25 ml NH4OAc (pH=7) in an oven dried 500 ml conical flask. The saturated solution was filtered in Buchner funnel and the final volume was made up to 100 ml by adding distilled water. Pretreatment of the soil extract was done by removing excess NH4OAc and organic matter from soil extract by complete evaporation of the aliquot, followed by treatment with aqua regia and second evaporation to dryness. The dried residue was dissolved in a quantity of distilled water equal to the original volume of the aliquot taken for the treatment. Five ml of the pretreated soil extract was taken in a china clay disc and was diluted to 100 ml with distilled water. Fifteen ml of NH4Cl + NH4OH buffer and 1-2 drops of masking agent and eriochrome black T (EBT) indicator were added to the diluted soil extract for determination of Ca+Mg. The
solution was titrated with 0.01N EDTA with continuous stirring until the colour changed from red to bright blue. Same procedure was followed for blank except 5 ml of distilled water was taken instead of Ca solution. For determination of Ca, 5 ml of the pretreated soil extract was taken in a china clay disc, followed by addition of 5 ml NaOH and pinch of ammonium purpurate. The solution was titrated against 0.01 N EDTA until the colour changed from pink to purple. Same procedure as earlier was followed for blank. The following formulas were used to determine the (Ca + Mg), Ca and Mg per 100 g soil and were expressed as ppm.

\[
\text{meq. of (Ca + Mg) or Ca per 100 g soil} = (V_2 - V_3) \times 0.01^* \times \frac{V}{V_1} \times \frac{100}{W}
\]

Where

- \(V_2\) = Titre value for sample
- \(V_3\) = Titre value for blank
- \(V_2 - V_3\) = Corrected titre value
- \(V\) = Volume of the extract made
- \(V_1\) = Volume of the aliquot taken for analysis
- \(W\) = Weight of the soil taken
- \(^*\) = Normality of EDTA

\[
\text{meq. of Mg/100 g of soil} = \text{meq. of (Ca + Mg)/100 g of soil} - \text{meq. of Ca/100 g of soil}
\]

3.4 Heavy metals/metalloid (Cr, Ni, Cu, Zn, As, Cd, Pb)

The heavy metals/metalloid present in the soil were digested with triple acid mixture and was estimated by following atomic absorption spectrophotometer (AAS) method (Jackson, 1967). One gram air-dried and sieved soil was mixed with 20 ml triple acid mixture (HNO\(_3\): HCl: H\(_2\)SO\(_4\)::1:2:4) in a digestion flask and digested by heating. The digestion was continued until the colour of the digested material became white and acid evaporated. Ten ml of HCl: H\(_2\)O mixture in 1:1 ratio was added and whole digested content was filtered through Whatman filter paper no. 42. Total volume of the filtrate was adjusted to 50 ml by adding distilled water. Heavy metal analysis was
done using AAS (Shimadzu, AA-7000 series). The following formula was used for the calculation of heavy metal concentrations in soil.

\[
\text{Heavy metal/metalloid (ppm) = A} \times 50^* \times 1000^{**}
\]

Where
- \(A\) = Concentration of heavy metal (mg/ml)
- \(^*\) = Volume of the filtrate (50 ml = 1 g soil)
- \(^{**}\) = Conversion factor to ppm (mg/Kg)

### 3.5 Determination of soil enzyme activity

#### 3.5.1 Dehydrogenase activity

2, 3, 5-triphenyl tetrazolium chloride (TTC) reduction technique of Casida (1977) was followed to estimate soil dehydrogenase activity. One gram of fresh soil was mixed with 100 mg of CaCO\(_3\) and 1ml TTC solution (1\%) in a test tube. The test tube was corked and incubated at 30 \(^\circ\)C for 24 h after proper vortexing. Then, the slurry was filtered using Whatman No. 1 filter paper and extracted with methanol. The final volume of the filtrate was adjusted to 50 ml by adding methanol. Absorbance of the filtrate was read at 485 nm using a UV-visible spectrophotometer (Systronics spectrophotometer 2202) and methanol extract as blank. Dehydrogenase activity was calculated with the help of a standard curve of triphenyl formazan in methanol and expressed in terms of mg formazan per gram air-dried soil per 24 hours.

#### 3.5.2 Urease activity

McGarity and Mayer’s (1967) method was used for the estimation of urease activity. Ten gram of fresh soil was treated with 1ml toluene in 100 ml volumetric flask. The flask was allowed to stand for 15 mins to allow penetration of toluene into soil. Ten ml potassium citrate-citric acid buffer (pH 7.0) and 5 ml of urea solution (10 \%) were added to the flask. For control, 10 ml distilled H\(_2\)O was added in place of urea solution. The flask was shaken well and incubated at 37 \(^\circ\)C for 3 h. After incubation period, distilled H\(_2\)O was added and the final volume was made up to 100 ml. Ammonia released due to urease activity was measured using indophenol blue method. For this, 0.5 ml of filtrate and 5 ml distilled H\(_2\)O was added and taken in 25 ml volumetric flask. The content of the flask was treated with 2 ml phenolate solution
and 1.5 ml of sodium hypochlorite solution. The final volume was adjusted to 25 ml using distilled H₂O and then, absorbance was read at 630 nm on a UV-visible spectrophotometer (Systronics spectrophotometer 2202). The released amount of NH₄⁺–N was calculated from a standard curve and expressed as NH₄⁺–N per gram air-dried soil per 3 hour. For control, same procedure was followed without soil.

3.5.2.1 Preparation of phenolate solution

*Phenol Solution:* Phenol weighing 62.5 g was dissolved in 20 ml of methanol. 18.5 ml of acetone was added to it and the final volume was adjusted to 100 ml by adding ethyl alcohol.

*NaOH Solution:* NaOH weighing 27 g was dissolved in 100 ml of distilled H₂O.

Phenolate solution was prepared fresh by mixing 20 ml each of phenol and NaOH solutions. The final volume was adjusted to 100 ml with distilled H₂O.

3.5.3 Phosphatase activity

Phosphatase activity of soil was estimated by following the method of Tabatabai and Bremner (1969). Air-dried and sieved (0.2 mm) 0.1 g soil was taken into 50 ml conical flask. To the flask, 4 ml of modified universal buffer (pH 6.5), 0.25 ml of toluene and 1 ml of 0.115 M p-nitrophenyl phosphate (PNP) were added and swirled properly before incubation at 37°C for 1 h. After incubation 1 ml of 0.5 M CaCl₂ and 4 ml of 0.5 M NaOH were added to the flask. The soil suspension was filtered using Whatman No. 1 filter paper and then the absorbance was read at 430 nm on UV-visible spectrophotometer (Systronics spectrophotometer 2202). For control, same procedure without soil was followed. The concentration of p-nitrophenol was estimated for determination of phosphatase activity using a standard curve of p-nitrophenol and expressed as moles of p-nitrophenol released per gram air-dried soil per hour.

3.6 Enumeration and identification of AM fungal spores

Spores of arbuscular mycorrhizal fungi were extracted from soil samples using slightly modified combination of wet sieving and decanting method (Gerdemann and Nicolson, 1963) and sucrose density gradient centrifugation (Daniels and Skipper, 1982). Twenty gram air-dried soil was suspended in 1000 ml of water and shaken for 10 mins and kept undisturbed for 1 h in order to allow heavier particles to settle down.
Care was taken not to disturb the spore structures. The soil suspension was decanted through a series of sieves of different sizes i.e 250 µm (1st), 125 µm (2nd), 60 µm (3rd) and 37 µm (4th). The sievings retained on the 1st and 2nd sieves were suspended in minimal amount of water and filtered using filter paper. Filter papers were transferred to petridishes and spores were counted and sorted based on their size, shape, and colour under stereomicroscope (Labomed CZM4 model; 10X×4X) using bamboo needle (15 cm long). The sievings retained on the 3rd and 4th sieves were washed and centrifuged at 2000 rpm for 5 mins. The supernatants were poured off and the respective pellets were resuspended in 40% sucrose solution and centrifuged at 2000 rpm for 1 min. The supernatants were sieved through 37 µm sieve. Spores were washed and observed under stereomicroscope as described above. The morphologically identical spores were used together (20-25 nos.) for preparation of diagnostic slide using polyvinyl-lacto-glycerol (PVLG) or PVLG+Melzer’s reagent (1:1 ratio) as mountants and observed under compound microscope (Labomed ATC2000 model;10X×40X magnification) using the method of Schenck and Perez (1990). Identification of AM spores were done based on size, shape, colour, wall structure, surface ornamentation, hyphal attachments and presence or absence of bulbous suspensor using online species descriptions of INVAM (International Culture Collection of Vesicular-Arbuscular Mycorrhizal Fungi; http://invam.caf.wvu.edu), http://www.amf-phylogeny.com, http://indexfungorum.org and published original species descriptions.

3.7 Storage and maintenance of AM fungal spores
Temporary storage of extracted AMF spores was done in Ringer’s solution (NaCl: 8.6g, KCl: 0.3g, CaCl₂: 0.33g distilled H₂O: 1L). For long term storage and maintenance monosporic and trap cultures were established.

3.7.1 Monosporic culture of AMF
For establishment of monosporic culture, soil and sand in 1:1 ratio was sterilized twice separately with a time gap of 24 h in polypropylene bags at 15 lb inch⁻² pressure for 20 min. Five maize seeds were allowed to germinate in a medium sized plastic pot filled with sterilized soil and sand (1:1) mixture. After germination seedlings were thinned to 3 per pot. The roots of maize seedling was inoculated with a healthy single
spore using a bamboo needle after separating and washing it with distilled water under dissection microscope. Establishment of root infection was confirmed after 30 days by collecting root pieces and examining them for colonization. Upon confirmation, successful cultures (soil and sand mixture with root pieces) were transferred to big pots (2.5 Kg). The spores of each isolate were identified and maintained for further investigation.

3.7.2 Trap culture of AMF

Trap cultures were established from fresh composite soil samples. *Allium cepa* and *Zea mays* were used alternatively as trap plants. The experiment was conducted under normal conditions.

3.8 Root colonization

Fine root samples collected from study sites were gently rinsed with tap water to remove adhering soil particles and were preserved in Formalin-Acetone-Alcohol (FAA) solution prior to root colonization assessment test. Root staining was done following the method of Kormanik and McGraw (1982) with slight modifications. Stored roots were washed properly in tap water and root segments of 1 cm length were placed in a 100 ml conical flask with KOH (10%) for clearing the cytoplasmic content and pigments. Root segments were kept in a conical flask and were boiled in water bath at 90-100 °C for 15 minutes. The hard roots, instead, were autoclaved at 15 psi for 10 minutes. The contents were poured off from flask on a sieve and washed well with tap water. The coloured root samples were bleached with freshly prepared alkaline H₂O₂ (3 ml NH₄OH; 30 ml 10 % H₂O₂; 567 ml H₂O) for 10-20 mins (until the colour of the roots became whitish). They were washed repeatedly with tap water to remove the traces of H₂O₂. The cleared and/or bleached root segments were acidified with 2% HCl for 5 minutes. They were stained with 0.05% (W/V) trypan blue and/or acid fuschin by incubating them overnight at room temperature. Trypan blue stained roots were destained for 1 h in lactic acid solution. The stained root segments were mounted on slide using lactophenol and examined under light microscope. The percent root colonization for estimation of mycorrhizal infection was calculated using the following formula:
Root colonization (%) = \frac{\text{Total number of colonized root segments}}{\text{Total number of root segments examined}} \times 100

3.9 Quantification of glomalin related soil protein (GRSP)

Glomalin, a glycoprotein produced by Arbuscular Mycorrhizal Fungi (AMF), was extracted by following the method described by Wright and Upadhyaya (1998) with slight modifications as done by Cornejo et al. (2008). Total Glomalin Related Soil Protein (GRSP), extracted from 1g air-dried soil, was mixed with 8 ml of 50 mM sodium citrate buffer (pH 8.0) and the mixture was autoclaved at 15 lb inch\(^{-2}\) pressure for 1 hr. Autoclaved samples were immediately centrifuged at 8000 x g (Remi Cooling Centrifuge, C-24 BL, India) for 20 minutes and filtered through Whatman No. 1 filter paper. The supernatant was then stored at 4 °C. The extraction procedure was repeated with the soil pellet until the straw coloured supernatant kept appearing. Supernatants from all the extraction cycle of a sample were combined and centrifuged again. The pellet was discarded and the final volume of extract containing the GRSP was measured and quantified using BSA standard curve (Bradford Protein Assay). The extracts were temporarily stored at 4 °C prior to quantification.

3.9.1 Bradford assay

GRSP was quantified using Bradford assay method. For this, standard curve of Bovine Serum Albumin (BSA) was prepared according to Bradford (1976). Stock solution was prepared by dissolving 10 mg BSA (HiMedia) in 10 ml of PBS buffer. The stock solution was diluted to get 0, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 µg/ml concentrations using phosphate buffer saline (PBS) having a final volume of 20 µl each. One milliliter of Bradford reagent was added to each standard dilution, vortexed and incubated constantly for 20 mins. Absorbance was measured at 595 nm using UV-visible spectrophotometer (Systronics spectrophotometer 2202) and the standard curve was prepared. Twenty microliter test sample was mixed with one milliliter Bradford reagent and the absorbance was read after proper mixing and incubation as done for standards. The O.D was then compared with the standard curve and concentrations were calculated using equation \( y = mx + b \) derived from the standard curve, where, \( y = \) absorbance at 595 nm (dependent variable), \( m = \) slope of line, \( x = \) protein concentration (independent variable) and \( b = Y \) intercept.
3.9.2 Heavy metal/metalloid sequestration by GRSP

Heavy metal/metalloid sequestration by GRSP was determined by following the method as described by Gonzalez-Chavez et al. (2004). The extracted GRSP was precipitated by slowly adding 3N HCl until the pH lowered to 2.5. The extract was kept overnight at 4°C. The precipitates were separated by centrifuging the content at 8000 x g (Remi Cooling Centrifuge, C-24 BL, India) for 20 minutes and redissolved in 100 mM sodium borate solution (pH 9.0). It was then dialyzed against deionized water and freeze-dried. The GRSP at the concentration of 5mg/ml was added with 100 ppm heavy metal/metalloid (Zn, Ni, Cu and As) separately and incubated for 24 h. The heavy metal precipitates were separated and acid digested in H₂O: HCl: HNO₃ (in 8:1:1 ratio; v/v/v). GRSP-heavy metal complex was determined by AAS.

3.10 Isolation of plant growth promoting rhizobacteria (PGPR)

Plant growth promoting rhizobacteria (PGPR) of the study sites were isolated by following dilution plate technique (Johnson and Curl, 1972). One gram composite and sieved (2 mm) soil sample was suspended in 10 ml of sterilized distilled H₂O and stirred constantly (150 rpm) in shaking incubator (Remi Shaking Incubator, India) at 28°C for 1 h to prepare stock suspension (10⁻¹ dilution). Ten fold dilutions of the stock suspension was serially prepared by pipetting 1 ml of previous dilution to 9 ml of dilution blanks of sterilized distilled H₂O each time for each dilution. One ml inoculum of the desirable dilutions was transferred to specific culture media viz. King’s B Agar (*Pseudomonas*), Pseudomonas Isolation Agar (*Pseudomonas*), Pikovskaya’s agar (Phosphate solubilizer), Ashby’s nitrogen free mannitol agar (*Azotobacter*), Rojo Congo agar (*Azospirillum*) and Jensen’s agar media (nitrogen fixing bacteria). The number of viable culturable bacterial cells present in soil was calculated in the form of colony forming units (CFU) using the following formula:

\[
\text{CFU/g dry soil} = \frac{\text{Average no. of colonies} \times \text{Dilution factor}}{\text{Dry weight of the soil}}
\]

3.11 Isolation of biosurfactant producing bacteria

Biosurfactant producing bacteria were isolated using liquid enrichment media containing crude oil as sole carbon source. One gram of composite soil sample of each site was added to Erlenmeyer conical flasks in triplicate containing 100 ml
mineral salt solution and nutrient broth (HiMedia, India) in 1:1 ratio. Crude oil collected from sivasagar oil field, Assam, India was separately sterilized by autoclaving and was mixed at 2% (w/v) as a sole carbon source in the liquid enrichment media. For each soil sample four flasks containing enriched media were prepared for 4 cycles of growth. The mineral salt solution used was composed of (g/l): KH$_2$PO$_4$ 0.7, K$_2$HPO$_4$ 0.7, MgSO$_4$$\cdot$7H$_2$O 0.7, NH$_4$NO$_3$ 1.0, NaCl 0.005, FeSO$_4$$\cdot$7H$_2$O 0.002, ZnSO$_4$$\cdot$7H$_2$O 0.002, and MnSO$_4$$\cdot$7H$_2$O 0.001. The pH of the salt solution was adjusted to 7.0±0.2 using electrical Cyberscan Eutech pH meter. The enriched flasks were incubated at 30°C in Rotary Shaking Incubator (Remi India) at 150 rpm for four days. Five milliliter of inocula from the first set of incubated flasks were subsequently transferred to second set of flasks containing blank enriched media and were maintained under same conditions. This process was repeated for 3rd and 4th set of blank enriched media flasks. Serial dilutions of the 4th sets of flasks were done and were plated on nutrient agar (HiMedia, India) plates by pour plate and streak plate method (Saikia et al., 2012).

3.12 Maintenance of bacterial isolates
Pure cultures of the isolates were prepared from the discrete colonies of the mixed culture using streak plate method (Cappuccino and Sherman, 2010). All the isolates were maintained for further investigation by subculture technique and subsequently preserving them at 4°C in Nutrient agar slants. Long term preservation was done at −20°C in cryovials with 15% (V/V) glycerol as cryopreservant.

3.13 Characterization of pure bacterial isolates
Characterization of the bacterial pure cultures was done based on the cultural, morphological and biochemical attributes as described by Cappuccino and Sherman (2010) and Bergey’s Manual of Systematic Bacteriology (Brenner et al., 2005; de Vos et al., 2009). The cultural attributes of the bacterial colonies like abundance of growth, size, form, margin, elevation, pigmentation etc were observed. Morphological and microscopic attributes of the bacteria were tested using Gram stain, spore stain (Schaffer and Fulton’s method) and capsule stain (Manewal’s method). KOH string test was also performed to validate the Gram stain (Smith and Hussey, 2005). A loopful of the culture (24 h old) was emulsified with a drop of 3% KOH on a clean
slide for 1 min. Development of string during lifting of loop confirms the Gram-negative strains. The following biochemical tests were performed for characterization of the isolates.

### 3.13.1 Catalase test

Catalase enzyme produced by certain groups of bacteria capable of detoxifying toxic \( \text{H}_2\text{O}_2 \), as an end product of aerobic respiratory pathway was tested. This enzyme catalyzes the breakdown of \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) and release free oxygen.

\[
\text{Catalase} \quad \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}_2
\]

This test was conducted by pouring 1 ml of \( \text{H}_2\text{O}_2 \) (3%) over 24 to 48h old bacterial culture incubated at 28\(^0\)C. Development of gas bubbles indicated positive test.

### 3.13.2 Amylase test

Amylase test also known as starch hydrolysis test was performed to detect amylase, an exoenzyme, produced by bacteria capable of hydrolysis of starch into simpler sugars. For amylase production or starch hydrolysis evaluation, the test bacteria were single streaked on starch agar plate (Nutrient Agar Media supplemented with 2% soluble starch) and incubated at 37 \(^0\)C for 48 h. The plates were flooded with iodine solution and excess of the solution was poured off. A clear zone around the colony indicated amylase production.

### 3.13.3 Lipase test

This test otherwise known as lipid hydrolysis test was performed to detect the exoenzyme lipase. The test bacteria were streaked on tributyrin agar plates (Nutrient Agar Media supplemented with 1% tributyrin) and were incubated at 37 \(^0\)C for 48 h. A clear zone around the colony indicated lipase production.

### 3.13.4 Casein hydrolysis test

It is also known as caseinase production test. The test bacteria were streaked on skim milk agar plates and incubated at 37 \(^0\)C for 48 h. A clear zone around the colony indicated hydrolysis of casein.
3.13.5 **Gelatin hydrolysis test**
This test was performed to detect the production of the exoenzyme gelatinase capable of gelatin hydrolysis (liquefaction).

\[
\text{Gelatin} + \text{H}_2\text{O} \xrightarrow{\text{Gelatinase}} \text{Polypeptides} \\
\text{Polypeptides} + \text{H}_2\text{O} \xrightarrow{\text{Gelatinase}} \text{Amino acids}
\]

Deep tubes of gelatin agar media were prepared and stab inoculated with test organisms. After incubation at 37 °C for 5 days, the tubes were kept at 4 °C for 15 min. The tubes that were liquefied at low temperature indicated gelatinase production and positive gelatin hydrolysis test.

3.13.6 **Oxidase test**
It was aimed to detect ability of bacteria to produce cytochrome oxidase enzyme involved in electron transport system. This test was performed by touching and spreading pure 48 h old bacterial culture on the oxidase disc (HiMedia, India) which was already impregnated with N, N-dimethyl-p-phenylenediamine oxalate (test reagent), ascorbic acid and α-naphthol. Development of blue to dark blue colour within 5-10 sec at 25-30 °C indicated positive test.

3.13.7 **Oxidation Fermentation (OF) test**
This test was performed to evaluate the capability of bacteria to metabolize glucose by aerobic respiration or fermentation. The test was performed by stab inoculating two tubes of Hugh and Leifson’s (1953) OF basal agar supplemented with sterile glucose solution (1% final concentration) with the test bacterial culture. One of the tubes was overlaid with 1 cm of sterile parafilm oil for ensuring the anaerobic condition. The tubes were incubated at 35 °C for 2-4 days depending on the rate of growth of the organism. Bacterial fermentation was indicated by the appearance of yellow colour in both tubes and oxidation was indicated by the appearance of yellow colour on the top of aerated tube only. Whereas, negative OF test was indicated by no colour change.
3.13.8 Indole production test

This test was performed to detect an enzyme tryptophanase which produce indole from tryptophan by reductive deamination reaction. Kovac’s method (1928) was used to detect indole from chemical reaction between indole and p-dimethylaminobenzaldehyde (DMAB) of the test reagent under acidic conditions. Bacterial isolates were inoculated in sterilized 5ml 1% tryptone broth test tubes and incubated for 48h at 35^0C. One milliliter of the Kovac’s reagent (HiMedia) was added to each tube and were mixed gently by shaking and were allowed to stand. Development of quinoidal compound (rosindole) appeared as cherry red colour ring indicating positive test for tryptophanase mediated indole production.

\[
\begin{align*}
\text{Tryptophan} & \xrightarrow{\text{Tryptophanase}} \text{Indole} + \text{Pyruvic acid} + \text{Ammonia} \\
\text{p-dimethylaminobenzaldehyde} + \text{Indole} & \xrightarrow{\text{HCl alcohol, Dehydration reduction}} \text{Quinoidal red-violet compound}
\end{align*}
\]

3.13.9 Methyl red and Voges-proskauer test

Methyl-Red (MR) test was performed to detect mixed acids and drop in pH due to fermentation of glucose present in the growth medium by the test bacteria. Voges-Proskauer (VP) test was used to detect acetoin (acetylmethylcarbinol), a fermentation pathway intermediates of glucose to 2, 3-butanediol due to difficulty in detection of the final product of the pathway. Each of the test isolates were inoculated in two 5ml MR-VP (buffered peptone-glucose broth) broth tubes and incubated at 35^0C for 48 h. For MR test, 5-7 drops of methyl red low pH (=4.0) indicator was added to a culture tube of a set. Development of red colour indicated positive reaction. While, for VP test Barritt’s method (1936) was followed. For this, 0.6 ml of barritt’s reagent A and 0.2 ml of barritt’s reagent B were added to second tube of the set. The solution was
gently shaken for 30 sec with caps off and left for 30 mins. Development of pink red colour indicated positive VP-test.

### 3.13.10 Citrate utilization test

The test was performed to detect enzyme citrase produced by test bacteria involved in utilization of citrate as carbon source. For this, Simmon’s citrate agar slants (pH 6.9) were inoculated with test bacteria and incubated at 35 °C for 2-5 days. Change in colour of the bromothymol blue incorporated media from green to blue indicated positive test.

\[
\text{Citrate} \rightarrow \text{Oxaloacetic acid + Acetic acid} \rightarrow \text{Pyruvic acid + CO}_2 \\
2\text{CO}_2 + 2\text{Na}^+ + \text{H}_2\text{O} \rightarrow \text{Na}_2\text{CO}_3 \\
\text{Alkaline pH} \rightarrow \text{Appearance of Blue Colour}
\]

### 3.13.11 Nitrate reduction test

This test was performed to detect enzyme nitrate reductase by test bacteria involved in the reduction of nitrates to nitrites or beyond it. For this, Trypticase nitrate broth tube was inoculated with test bacterium and incubated at 37 °C for 2-5 days. Five drops of sulfanilic acid (Sol\(^a\). A) and \(\alpha\)-naphthylamine (Sol\(^b\). B) were added to the incubated tubes. Immediate appearance of cherry red colour indicated positive nitrate reduction test. Appearance of red colour after adding a pinch of zinc powder, which reduced nitrate to nitrite, confirmed negative nitrate reduction test. No change in colour despite addition of zinc powder indicated reduction beyond nitrites.
3.13.12 Litmus milk test

This test was performed to evaluate the enzymatic transformations of different milk substrates into varied metabolic end products. For this, two tubes of Litmus milk broth medium were prepared, which contained lactose, casein and litmus components as substrate. One of the tubes was inoculated with the test bacterial culture and both tubes were incubated at 37 °C for 2-5 days. Possible observations and inferences of this test are summarized in the table below.

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue/Unchanged</td>
<td>Alkaline</td>
</tr>
<tr>
<td>Pink</td>
<td>Acid</td>
</tr>
<tr>
<td>Pink band + White media</td>
<td>Acid and reduction</td>
</tr>
<tr>
<td>Pink band + Curd</td>
<td>Acid, reduction and curd</td>
</tr>
<tr>
<td>Pink band + Curd with fissures</td>
<td>Acid, reduction, curd and gas</td>
</tr>
<tr>
<td>Purple band + White media</td>
<td>Litmus reduction</td>
</tr>
<tr>
<td>Deep purple band + Whey like brownish translucent media</td>
<td>Casein proteolysis</td>
</tr>
</tbody>
</table>

3.13.13 Triple sugar iron (TSI) test

This test was aimed to evaluate the patterns of fermentation capability of triple sugar (Lactose, Sucrose and Glucose) and H₂S production by test bacterial culture. For this, Triple sugar iron agar slants were prepared and inoculated with test bacterial culture. Observations were made after incubation at 37 °C for 24 h. The expected observations and inferences of the test are summarized in the table below:

<table>
<thead>
<tr>
<th>Appearance (slant + butt)</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fermentation</td>
</tr>
<tr>
<td>Red (alkaline) + yellow (acid)</td>
<td>Glucose</td>
</tr>
<tr>
<td>Yellow + Yellow</td>
<td>Glucose, Lactose and/or Sucrose</td>
</tr>
<tr>
<td>Red + Red</td>
<td>Nil</td>
</tr>
<tr>
<td>Yellow + Yellow with bubbles</td>
<td>Glucose, Lactose and/or Sucrose</td>
</tr>
<tr>
<td>Red + Yellow with bubbles</td>
<td>Glucose</td>
</tr>
<tr>
<td>Red + Yellow with bubbles, Black precipitate</td>
<td>Glucose, Lactose and/or Sucrose</td>
</tr>
<tr>
<td>Yellow + Yellow with bubbles, Black precipitate</td>
<td>Glucose, Lactose and/or Sucrose</td>
</tr>
<tr>
<td>Red + Yellow, Black precipitate</td>
<td>Glucose</td>
</tr>
<tr>
<td>Yellow + Yellow, Black precipitate</td>
<td>Glucose, Lactose and/or Sucrose</td>
</tr>
</tbody>
</table>
3.13.14 Urease test
This test was performed to detect urease enzyme, which acts on urea and produce alkaline ammonia. Urea broth tubes containing phenol red pH indicator were prepared and inoculated with test bacterial culture excluding the control tube. The tubes were then incubated at 37 °C for 48 h. Development of pink color indicated positive urease test.

3.14 Plant growth promoting characteristics
The indigenous bacterial isolates were evaluated for their potential for plant growth promotion.

3.14.1 Indole acetic acid (IAA)
Indole acetic acid (IAA) was detected by following the method as described by Rahman et al. (2010). Bacterial strain was inoculated in 50 ml Luria-Bertani broth medium (HiMedia) for 72h at 28°C. One ml of the supernatant obtained by centrifuging at 8000 rpm for 15 min was added with one drop of orthophosphoric acid and 2ml of Salkowski’s reagent (50 ml, 35% of perchloric acid and 1 ml of 0.5 M FeCl₃ solution). The solution was kept in dark and incubated at room temperature for 30 mins. Development of pink colour indicated IAA production.

3.14.2 Production of ammonia (NH₃)
Ammonia (NH₃) was detected and quantified by following the method of Demutskaya and Kalinichenko (2010). Ten milliliter of peptone water tubes were prepared and inoculated with freshly grown bacterial strain. The inoculated broth medium was incubated at 28 °C for 72h. One milliliter of both culture supernatant and Nessler’s reagent were mixed and the final volume was adjusted to 10 ml with distilled H₂O. Development of brown to yellow colour indicated ammonia production.

3.14.3 Production of hydrogen cyanide (HCN)
Hydrogen cyanide production (HCN) was tested by the method as described by Ahmad et al. (2008). Nutrient agar plates supplemented with glycine (4.4 g l⁻¹) was inoculated by streaking with freshly grown bacterial strain. Sterile filter paper was soaked in the solution of 0.5% picric acid and 1% Na₂CO₃. The picric acid impregnated and saturated filter paper was then placed on the inner side of the upper
lid of the petriplates. The test and control plates were sealed with parafilm and incubated at 28 °C for 72 h. Development of orange to red colour indicated HCN production.

3.14.4 Production of salicylic acid ($\text{C}_6\text{H}_4(\text{OH})\text{COOH}$)
Salicylic acid ($\text{C}_6\text{H}_4(\text{OH})\text{COOH}$) production was tested and quantified by the method of Meyer and Höfte (1997). Fifty milliliter of succinate medium was inoculated with freshly cultured bacterial strain and incubated at 28 °C for 48 h in rotary shaker at 150 rpm (Remi, India). Four milliliter of the supernatant was obtained by centrifuging the broth culture at 8000 rpm for 15 mins and cell free supernatant was acidified with 1N HCl (pH=2). Salicylic acid was extracted from acidified supernatant in chloroform ($2\times2$ ml). One milliliter of the extract was mixed with 5µl of 2M FeCl$_3$ and 3ml of distilled H$_2$O. Appearance of purple colour indicated salicylic acid production.

3.14.5 Phosphate solubilization assay
This assay was conducted to estimate the activity of phosphatase produced by test bacteria (Pikovskaya, 1948). Culture broth with approximately $10^8$ cfu/ml was spot inoculated in triplicate on the same Pikovskaya agar plate using sterilized toothpick and incubated for 72 h at 37 °C. The clear zone around the colony indicated phosphate solubilization.

3.15 Heavy metal/metalloid resistance test
Bacterial resistance to heavy metals was determined by both turbidity and agar dilution method as described by Oyetibo et al. (2010) and Aleem et al. (2003). In turbidometric method different dilution (1-20 mM) of heavy metal/metalloid salts NiCl$_2$, 6H$_2$O, ZnCl$_2$, CuSO$_4$.5H$_2$O and NaAsO$_2$ amended nutrient broth media were prepared and sterilized in test tubes. Fifty µl of the 1 O.D bacterial culture was inoculated in already prepared heavy metal amended media and incubated at 30 °C for 3 days and the incubation was extended up to 7 days. Growth media without inoculation was used as control. Bacterial growth was measured by taking absorbance at 600 nm in UV-VIS spectrophotometer. The lowest heavy metal/metalloid concentration at which bacteria failed to grow was considered as minimum inhibitory
concentration (MIC). In order to verify the turbidometric results, agar dilution method was used by preparing nutrient agar media amended with different dilutions of heavy metals/metalloid as mentioned above. The heavy metal/metalloid amended nutrient agar plates were inoculated with 1 O.D bacterial culture by streaking and were incubated as described above in case of turbidometric method. Bacterial culture capable to grow at 1 mM Ni$^{2+}$, Zn$^{2+}$, Cu$^{2+}$ and As$^{3+}$ were considered resistant.

3.16 Production of biosurfactant

Bacterial isolates were screened for their ability to produce surface active compounds (biosurfactant/bioemulsifier) by performing different tests based on the reduction of interfacial tension and emulsification.

3.16.1 Drop collapse assay

Drop collapse test was performed as described by Čipinytė et al. (2011). Clean microscopic slide was coated with crude petroleum oil and 10 µl of the culture supernatant was placed on the oil coated slide. Larger diameter of the test drop compared to the distilled H$_2$O indicated the presence of surface active compounds.

3.16.2 Emulsification assay ($E_{24}$)

The emulsifying capacity of the culture supernatant was evaluated by emulsification index ($E_{24}$) as described by Cooper and Goldenberg (1987). Two ml each of culture supernatant and kerosene oil were mixed in flat bottom screw cap vial. The mixed liquid was vortexed for 5 mins and kept undisturbed for 24 hrs. The ratio of the height of the emulsified layer and the total height of the mixed solution was measured to determine the emulsification index ($E_{24}$ values). The formula used to calculate the $E_{24}$ value in percentage is as follows:

$$E_{24}(\%) = \frac{\text{Height of the emulsified layer}}{\text{Total height of the solution}} \times 100$$

3.17 Kinetics of biosurfactant production

Kinetics of surface active compounds produced by bacterial strains was studied by inoculating 500 ml of nutrient broth media with 1% (1 OD$_{600}$) fresh test bacterial suspension and incubating them at 28 $^\circ$C for 30 days in rotary shaking incubator (150 rpm). During the investigation, under aseptic conditions, samples were removed from
broth culture at every 24 h to assess the surface active compounds production for cell free supernatant (CFS) using E24 index as described earlier.

3.18 Extraction of biosurfactant

The crude biosurfactant was extracted by following acid precipitation and organic solvent extraction method (Wei et al., 2005). Bacterial cells were removed from the culture broth after optimum incubation time of the respective isolates by centrifuging at 10,000 x g, for 15 min at 4 °C. The cell free supernatant (CFS) was acidified with 6N HCl (pH 2.0) to obtain the isoelectric point in order to decrease solubility of protein and lipid containing biosurfactants. The resultant solution was stored overnight at 4 °C to enhance the precipitation of desired molecules. Crude biosurfactant was extracted thrice using chloroform: methanol (2:1, V/V) after acidification at room temperature. Organic solvent of the extract was evaporated using rotary evaporator at 40 °C to obtain crude biosurfactant as viscous brown coloured material. It was weighed and expressed as g/L.

3.18.1 Properties of crude biosurfactant

3.18.1.1 Determination of critical micelle concentration (CMC)

Critical micelle concentration (CMC) of crude biosurfactant was measured for determining surfactant concentration corresponding to the concentration at which micelle formation is initiated. A series of crude biosurfactant dilutions were prepared in distilled water (0-250 mg/L) and surface tension was measured respectively using tensiometer (model K1, Kruss Optronic, Germany). The concentration beyond which no more reduction of surface tension was observed due to formation of micelles was considered as CMC (Khopade et al., 2012).

3.18.1.2 Metal/metalloid chelating activity of the biosurfactant

Heavy metals/metalloid were added to crude biosurfactants at 500 ppm concentration and incubated overnight. The solution was centrifuged at 10,000×g for 10 minutes to separate metal/metalloid-biosurfactant precipitate. Heavy metal concentrations that remained in the supernatant were measured using Atomic Absorption Spectrophotometer (AAS).
3.18.3 Characterization of biosurfactant

The biosurfactant was characterized to determine their nature and composition as described by Saikia et al. (2012) and Patowary et al. (2014) using biochemical tests, thin layer chromatography (TLC) and infra-red spectral analysis.

3.18.3.1 Biochemical analysis

The biochemical analysis of the crude biosurfactant was done according to the method of Sawhney and Singh (2000). Carbohydrate molecule in the crude biosurfactant was determined by anthrone test. Five ml each of culture supernatant and anthrone reagent were mixed properly. Development of blue-green colour indicated the presence of carbohydrate. The presence of amino acids and proteins were determined by ninhydrin test. Five drops of ninhydrin was mixed with 5 ml of culture supernatant and kept in boiling water bath for 5 min. Development of purple colour (Ruheman’s complex) indicated their presence. Lipid content was determined by saponification test. Five ml culture supernatant was mixed with 2 ml NaOH (2%) followed by vortexing for 1 min. Development of foam indicated presence of lipid.

3.18.3.2 Determination of Rf-value

Crude biosurfactant was analyzed by thin layer chromatography (TLC). The dried crude biosurfactant was dissolved in chloroform: methanol (2:1) in a concentration of 10 mg/ml (Saikia et al., 2012). TLC plate was prepared using silica gel G (SRL, India). The powder was mixed with water and the slurry was spread on the clean and dried glass plate keeping the thickness of the gel not more than 0.25 mm. The air dried plate was activated by oven drying at 110 °C for 30 min. For analysis, 2 µl of the sample was spotted onto the TLC plate about 1.5 cm from the bottom edge of the plates. The spot was air dried and placed into the TLC tank filled with mixture of chloroform: methanol: acetic acid: water (25:15:4:2, v/v/v/v). The tank was closed with glass cover. The plate was removed, when the solvent front moved 3/4th distance of the plate. The plate was dried and then placed in iodine chamber for few minutes before observation of the chromatogram of the crude biosurfactant. The retention factor (Rf) value was calculated using the following formula:

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$
3.18.3.3 Fourier transformed infrared spectroscopy (FTIR) analysis

Fourier transformed infrared spectroscopy (FTIR) was used for preliminary study and overall chemical nature of the extracted biosurfactant by exploring the functional groups and chemical bonds. In this technique, absorption of IR frequencies of a sample was measured. IR radiation does not have enough energy to induce electronic transitions as was observed with UV radiation. However, IR is absorbed by compounds with small energy differences in the possible vibrational and rotational states. Absorption of IR by a molecule, the vibrations or rotations, must cause a net change in the dipole moment of the molecule. Thus, IR spectroscopy elucidates the functional groups within molecule of a sample since absorption of IR frequency varies with different functional groups. Each molecule has a typical spectrum often referred as the fingerprint. The molecule was identified by referring the absorption peak to a data bank of spectra. The purified biosurfactant was analyzed by FTIR spectrophotometer (Shimadzu, Japan) by scanning it in the range of 4000-400 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\) using KBr plate of 0.26 mm thickness.

3.19 Mass multiplication of bioinoculants

3.19.1 AM Fungi

The inocula of AM fungi were prepared by mixing the potted soil with chopped roots of maize (Zea mays) and onion (Allium cepa) of single spore culture. Approximately, 200 spores/100 grams were considered as potent inoculum for application. Inoculum was placed near the roots of germinated seedling three times at an interval of 30 days.

3.19.2 PGPR

The desired bacteria were cultured in nutrient broth media at 28 °C for 2 days and centrifuged at 8000 rpm for 10 minute. The pellet was then suspended in sterilized distilled water to obtain a final bacterial density of 3\(\times\)10\(^6\) cfu ml\(^{-1}\). The bacterial suspension was applied to the soil adjacent to the main root system by soil drenching method @ 3 ml per pot three times at an interval of 30 days.

3.20 Screening of PGPR and AMF by biometric growth parameters

The potential AM fungal isolates and PGPR strains were further screened based on the biometric growth parameters of maize (Zea mays). The maize seeds were surface
sterilized with 0.5 % sodium hypochlorite (NaClO) solution for 5 min and subsequently washed several times with distilled water and were incubated at 28 °C for 48 h. Ten seeds were transferred to each of 63 pots and after germination thinned to three. All together 10 AM fungal strains (based on density) and 10 PGPR strains were inoculated separately in 3 replicates as described above. The plants were grown under normal temperature and day-light conditions for 12 weeks.

3.20.1 Root and shoot length
The shoot and root length were measured after the growth period using the centimeter scale. The complete plant with intact shoot and root was removed by loosening the soil carefully from the root system. The shoot length of the plants was measured from the base of the shoot to the longest leaf tip of the plant. The roots were also measured from the base to the tip of longest root.

3.20.2 Root and shoot weight
The fresh weight of shoot and remaining root were measured using digital balance. The shoots and roots were rinsed with tap water and then deionized water and weighed after oven drying at 70 °C for 48 h (Wang et al., 2007).

3.20.3 Chlorophyll content
Dimethyl sulphoxide (DMSO) extraction technique of Hiscox and Israelstam (1979) was used for the extraction of total leaf chlorophyll. One gram of fresh leaves from each treatment was chopped and placed in a test tube containing 10 ml of DMSO. The tube was incubated at 65 °C for 3 h and absorbance was read at 645 nm and 663 nm spectrophotometrically (UV-VIS Systronics Spectrophotometer 2202) by transferring 3 ml of the chlorophyll extract to a cuvette against DMSO blank. Arnon’s (1949) equation was used for calculation of total chlorophyll:

\[
\text{Total Chlorophyll (mg/g) = 0.0202 (A}_{645} + 0.00802 (A}_{663})
\]

3.20.4 AM fungal spore density
AM fungal spore density was determined using the method of Gerdemann and Nicolson (1963) as described under section 3.6. Root colonization was also studied to evaluate the AM fungal root colonization by the method as discussed under section 3.8 (Kormanik and McGraw, 1982).
3.21 Evaluation of screened AMF and PGPR strains under field conditions

The screened AMF and PGPR strains based on in-vitro and biometric growth parameters along with metallophyte (Zea mays) were evaluated for alleviation of soil heavy metal toxicity. Finally, three strains each of AMF and PGPR were used along with maize (Zea mays) as a test plant for pot experiment under net house conditions. The experiment was set in a complete randomized block design and five replications per treatment were considered. Pots added with heavy metals but without any bioinoculants acted as control.

3.21.1 Preparation of soil

Soil was collected from the forest near Department of Botany, Gauhati University, Guwahati and all the organic debris including roots were removed. The sieved soils were packed in polypropylene bags (2.5 Kg bag$^{-1}$) and sterilized as described under section 3.7.1. The sterilized soils were then transferred into pots (2.5 Kg pot$^{-1}$) and heavy metal/metalloid salts [NiCl$_2$. 6H$_2$O, ZnCl$_2$, CuSO$_4$.5H$_2$O and NaAsO$_2$] were added to the soil and mixed properly. The pots were kept undisturbed for 1 month to saturate heavy metals in the soil in the net house of Department of Botany, Gauhati University. Maize seeds were surface sterilized and sown in sterilized soil: sand mixture (1:1) for germination. After germination, three seedlings of equal height were transplanted into heavy metal/metalloid treated/ammended pots and all the bioinoculants were added as per the experimental design. The first dose was applied on the day of transplantation and the two subsequent applications at an interval of 30 days each from the day of first application. Bioinoculants were added thrice during the entire period of 12 weeks for which the experiment was continued. The potted plants were watered twice a week and utmost care was taken to prevent the leakage of water from the pot.

3.21.2 Mycorrhizoremediation of Heavy metals/metalloid

The shoots and roots were separated and washed with 0.01M EDTA followed by distilled water to remove any nonspecifically bound heavy metals. The shoots and roots samples were dried at 70 °C for 48 h and dry weight was determined. The oven dried samples were ground to approximately 0.5 mm size for heavy metal analysis.
Then, 30 mg of the each sample was wet digested in a mixture of concentrated HNO$_3$ and HClO$_4$ (3:2, V/V) (Wang et al., 2007). Heavy metal/metalloid (HMM) concentrations were determined using AAS (Shimadzu, AA-7000). The heavy HMM uptake was calculated following the formula as referred below. The change in HMM uptake percentage was calculated based on the mean heavy metal uptake by inoculated and uninoculated plants.

Change in HMM (uptake %) 
\[
= \frac{\text{HMM uptake of inoculated plants} - \text{HMM uptake of uninoculated plants}}{\text{HMM uptake of uninoculated plants}} \times 100
\]

HMM extraction efficiency (HEE) was calculated based on the ability of the root to transport HMs to shoot.

\[
\text{HEE (μg g}^{-1}) = \frac{\text{Shoot HMM uptake}}{\text{Root dry weight}}
\]

3.22 Molecular characterization

Molecular characterization of potent bacterial isolates was carried out using 16S rDNA gene sequence to authenticate the result obtained by cultural, microscopic and biochemical tests.

3.22.1 Preparation of TE buffer (Tris-EDTA buffer)

Hundred ml of TE buffer was prepared by adding 1 ml of 1M Tris HCl and 0.2 ml of 0.5 M EDTA in 98.8 ml of double distilled H$_2$O. Fifty ml stock solution of 1M Tris HCl was prepared by dissolving 7.88 g of Tris HCl (HiMedia) in 25 ml of double distilled water (pH 8.0) and the final volume was made to 50 ml by adding double distilled H$_2$O. Similarly, 0.5 M EDTA was prepared by dissolving 14.612 g EDTA (HiMedia) in 50 ml double distilled H$_2$O and pH was adjusted to 8.0 with NaOH. The final volume was made to 100 ml by adding double distilled H$_2$O. The stock solutions were autoclaved and stored at room temperature.

3.22.2 Preparation of 10% SDS

Five gram dodecylsulphate sodium salt (HiMedia) was dissolved in autoclaved double distilled H$_2$O and the final volume was made to 50 ml.
3.22.3 Preparation of 5M NaCl
NaCl (HiMedia) (14.61 g) was dissolved in 40 ml double distilled H₂O. The volume was adjusted to 50 ml, then autoclaved and stored at room temperature.

3.22.4 Preparation of CTAB/NaCl solution (10% CTAB in 0.7 M NaCl)
NaCl (4.1 g) was dissolved in 80 ml distilled H₂O and 10 g CTAB was slowly added to it with mild heating and stirring. Final volume was adjusted to 100 ml.

3.22.5 Preparation of TAE (Tris-Acetate-EDTA) buffer
3.22.5.1 50X TAE buffer
Tris base (121 g) was dissolved in 300 ml of distilled water and 28.55 ml of glacial acetic acid was added to it. 9.3 g EDTA was dissolved in 100 ml distilled water. The final pH was adjusted to 8.0 using NaOH. TAE buffer (50X) was prepared by mixing both the solutions and the final volume was made up to 500 ml. The autoclaved solution was stored at room temperature.

3.22.5.2 1X TAE buffer
The final (1X) working concentrations of Tris-acetate and EDTA were 0.04M and 0.001 M respectively. 1X TAE was prepared by adding 10 ml 50X TAE buffer to 490 ml sterile distilled water.

3.22.6 Ethidium bromide (EtBr)
Ethidium bromide (10 mg/ml) was prepared by dissolving 10 mg of EtBr in 1 ml of distilled water.

3.22.7 Isolation of genomic DNA
The bacterial genomic DNA was isolated by following the method of Wilson (1997). Twenty four hour old bacterial culture (1.5 ml) was transferred into 2.5 ml micro centrifuge tube and was centrifuged at 6000 rpm for 2 min. The supernatant was discarded and pellet was resuspended in 567 µl TE buffer. Thirty µl of 10% SDS and 3 µl of proteinase K (20 mg ml⁻¹, HiMedia) were added to this mixture and incubated at 37 °C for 1 h. Hundred µl of 5M NaCl was added after completion of incubation period and was mixed thoroughly. Thereafter 80 µl of CTAB/NaCl solution was added and after proper mixing it was incubated for 10 min at 65 °C. Equal volumes (0.750 ml) of Chloroform and isoamyl alcohol (HiMedia) were added and were
thoroughly mixed by mild vortexing. It was centrifuged at 12000 rpm for 5 min. The supernatant was transferred to fresh micro centrifuge tube leaving behind the white interface. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the separated supernatant and mixed thoroughly by spinning in a microcentrifuge for 5 min. The supernatant was transferred to fresh tube and chilled isopropanol was added to precipitate nucleic acids. The tube was shaken back and forth gently until a white DNA precipitate became clearly visible. Short spin was performed to pellet the DNA and it was washed with 70% ethanol to remove residual CTAB. The pellet was air dried properly.

3.22.7.1 Purification of genomic DNA
The extracted genomic DNA was resuspended in 100 µl TE buffer and incubated at 37 °C for 30 min after addition of 3 µl RNase (20mg/ml). The sample was thereafter re-extracted with equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) and RNA free DNA was precipitated with chilled isopropanol as described above. The quantity and quality of DNA was tested both spectrophotometrically and electrophoretically using 0.75% agarose gel.

3.22.7.1.1 Measurement of DNA concentration
The concentration of DNA was determined spectrophotometrically using UV-VIS spectrophotometer (Systronis 2202). Absorbance of the DNA sample was read at 260 nm and 280 nm. The following relations were used for quantification:

1 O.D at 260 nm for dsDNA= 50ng/µl

The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA samples. The ratio of 1.8 (OD_{260}/OD_{280}) is accepted as pure DNA sample. Significantly lower ratio indicated presence of protein, phenol or other contaminants which are also absorbed strongly at 280 nm.

3.22.7.1.2 Agarose gel electrophoresis
The agarose gel electrophoresis was used to know the DNA size and presence or absence of shearing of DNA samples. For this, 0.75 % agarose gel was prepared in 1X TAE buffer in a clean and dried 250 ml conical flask by melting in microwave oven. The molten agarose was allowed for cool down to 60 °C and ethidium bromide (EtBr)
was added carefully at a concentration of 0.5 µg/ml in the gel. Gel was poured on the gel casting tray with properly placed comb and allowed to solidify for 25 minutes. 5 µl of the sample was mixed with 2 µl of 6X gel loading buffer and loaded in the well of prepared gel. 2 µl of Marker (1Kb) was mixed with 2 µl of 6X gel loading buffer and 3 µl of water and then loaded in a well at one side of the gel. The gel was run at constant current 90 mA and voltage 80 volt (Bio-RAD Power Pac 3000). The run was continued until the bromothymol blue migrated an appropriate distance. The gel was then observed in Gel Documentation System (Bangalore Genie) for documentation.

### 3.22.8 PCR amplification

The partial 16S rDNA gene was amplified by polymerase chain reaction (PCR) using Thermal Cycler (Bio-RAD, C1000). The components of PCR master mix (50 µl) used are mentioned in Table 4:

#### Table 4: Concentration of PCR mastermix.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Components</th>
<th>Final conc.</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Water</td>
<td>-</td>
<td>38.7</td>
</tr>
<tr>
<td>2.</td>
<td>10X buffer with 25mM MgCl₂</td>
<td>1X</td>
<td>5</td>
</tr>
<tr>
<td>3.</td>
<td>25mM dNTPs</td>
<td>200 mM each</td>
<td>0.4</td>
</tr>
<tr>
<td>4.</td>
<td>Taq polymerase</td>
<td>2.5U</td>
<td>2.5</td>
</tr>
<tr>
<td>5.</td>
<td>Forward primer 27f</td>
<td>0.4µM</td>
<td>0.2</td>
</tr>
<tr>
<td>6.</td>
<td>Reverse primer 1492r</td>
<td>0.4µM</td>
<td>0.2</td>
</tr>
<tr>
<td>7.</td>
<td>DNA template</td>
<td>0.1-10 ng</td>
<td>3</td>
</tr>
</tbody>
</table>

Total Volume 50 µl

Two sets of universal primers (27f and 1492r; 1406f and 155r) were used for amplification of 16s rDNA gene sequence. Primers 27f and 1492r, however, were standardized and were used for the final sets of experiment based on the size of the DNA bands in agarose gel.

#### Table 5: Primers used for PCR amplification reaction for 16s rDNA gene

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Primer code</th>
<th>Primer sequence (5'-3')</th>
<th>% GC</th>
<th>TM</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>27f</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>50.0</td>
<td>51.8</td>
<td>Devereux and Wilkinson, 2004</td>
</tr>
<tr>
<td>2.</td>
<td>1492r</td>
<td>TACGGYTACCTTGTTACGACTT</td>
<td>43.2</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>
The temperature profiles used were initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 48 °C for 30 sec and extension at 72 °C for 1 min. Final extension was done at 72 °C for 7 min and then reaction chamber temperature was allowed to come down to 4 °C before the removal of amplified product. The amplification was verified by gel electrophoresis. The PCR product was purified by using HiPurA™ PCR Product Purification Kit (HiMedia, India).

3.22.9 Sequencing of 16S rDNA gene and Phylogenetic analysis
The PCR product was sequenced bi-directionally in Xcelris Laboratory, Ahmedabad. The DNA sequence was analyzed using software MEGA 6 by comparing to chromatogram sequence. After proper editing, if necessary, sequence was converted to FASTA format and BLAST to NCBI database for identification of the organism based on the homology of sequence. The significance of BLAST were tested by e value (expect value) generated by BLAST family of search algorithm. Phylogenetic tree construction based on 16S rDNA sequences was carried out using Neighbor joining method. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Divergence times for all branching points in the topology were calculated with the RelTime method (Tamura et al., 2012) using the branch lengths contained in the inferred tree. Evolutionary analyses were conducted in MEGA 6 (Tamura et al., 2013).

3.23 Data Analysis
Data were analyzed by matrix correlation (r^2) using SPSS 16 version software wherever required. Correlation analysis was done to evaluate the relatedness of different variables and subsequently, the direction and level of significance. Relative abundance (RA) and occurrence frequency (OF) as described by Dandan and Zhiwei (2007) were used to analyze the data related to AM fungal population. Two-way Analysis of Variance (ANOVA) was performed to evaluate the significance of variation as influenced by age of soil, season and their interactions using SPSS 16.0 version. ANOVA was also performed to evaluate the significance of treatments in alleviation of heavy metal toxicity. Principal Component Analysis was done using XLSTAT software and then biplot ordination diagram was prepared in order to
visualize the influence of disturbance caused by open cast coal mining on physico-chemical and biological properties of soil. Ordination analysis was also performed using AM fungal spore density and species data.