CHAPTER 3

MATERIALS AND METHODS

3. Materials and methods

3.1. Collection of nodule sample

For characterizing the diversity of rhizobia associated with legume plants of Assam, two cultivated and two wild leguminous plant was selected. The cultivated legumes were *Lablab purpureus* L. and *Cajanus cajan* L.. Both the selected plants were agriculturally very significant and cultivated by the peoples of Assam since time immemorial. The wild legumes were *Crotalaria pallida* L . and *Mimosa pudica* L. In the field, the legumes were identified using botanical key and plants were uprooted using a knife, avoiding detaching secondary roots from the plant, as nodules may be found on lateral roots as well as the tap root. Carefully the nodules were excised using a blade and placed in the vials containing silica gel. The nodules were transported to the laboratory in a cooler.

3.1.1. Description of selected plants

I. *Lablab purpureus* L.: *L. purpureus* (synonym *Dolichos lablab* L.), is a summer growing fodder legume. It is a vigorously trailing herbaceous plant and serves as a vegetable, herbal medicine, forage and green manure (Cameron, 1988).

Figure 4. *Lablab purpureus* plant

(Source:http://www.tropicalforages.info/key/Forages/Media/Html/images/Lablab_purpureus/Lablab_purpureus_10.jpg)

II. *Cajanus cajan* L.: *C. cajan* is one of the important pulse crops and a very popular food in developing tropical countries. It is one of the major legumes of the tropics among different pulses in respect of area and production.

Figure 5. *Cajanus cajan* plant

(Source:http://www.tropicalforages.info/key/Forages/Media/Html/images/Lablab_purpureus/Lablab_purpureus_10.jpg)

III. *Crotalaria pallida* : *C. pallida* is a wild leguminous plant, widely distributed in almost all the parts of India. *C. pallida* is an erect shrub, annual or short-lived perennial herb of 1.5 m or more tall.

Figure 6. *Crotalaria pallida* plant

(Source:http://herbariaold.plants.ox.ac.uk/vfh/image/imgs/604/500_crotalaria_pallida%5bgr904%5dgr20062b.jpg)

IV. *Mimosa pudica* L.: *M. pudica* is a creeping annual or perennial herb, widespread in tropical, subtropical and temperate areas of the world. Similar to the previous 3 legume plants it also belongs to family *Fabaceae.* *M. pudica* plant is well known for the movement of its leaves. The compound leaves fold inward when touched and re-open a few minutes later.

Figure 7. *Mimosa pudica* plant
(Source:http://www.biodiversityexplorer.org/plants/fabaceae/images/Mimosa-pudica,-11983,-Verulam,-Nov-2010_0149_419h.jpg)

3.1.2. Sample collection sites

Root nodules of the selected plants were collected from 8 different sites of Assam (Cachar, Karimganj, Hailakandi, N.C.Hills, Kamrup, Kokrajhar, Tezpur and Sivasagar). The selected sites belong to 3 different geographical regions of Assam namely, Barak valley, Brahmaputra valley and N. C. Hills region. Cachar, Karimganj and Hailakandi,from the Barak valley region. Sivasagar, Tezpur, Kamrup and Kokrajhar are included under the Brahmaputra valley region. N.C. Hills region is located in between the Barak and Brahmaputra valley.

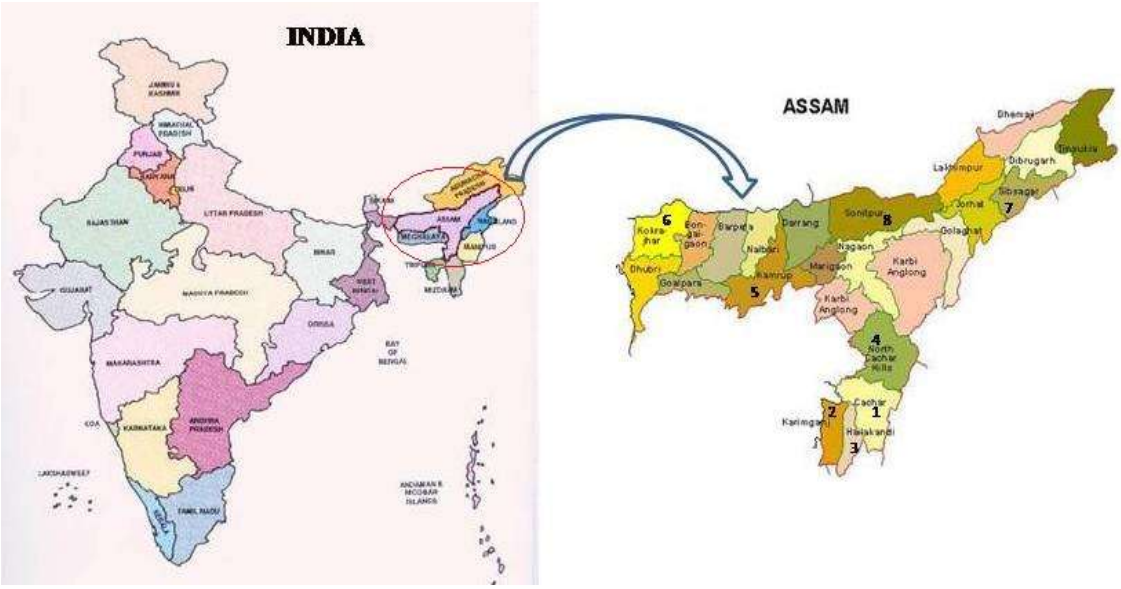

Figure 8: Sample collection sites

Table 1. Nodule sample collection sites

State	Location in Fig. 9	Collection sites	Latitude/Longitude	Date of collection
Assam	1	Cachar	25.08^0N-82.91^0E	1-15 Dec. 2012
	2	Karimganj	24.87^0N-92.35^0E	15-30 Dec. 2012
	3	Hailakandi	24.68^0N-92.57^0E	1-15 Jan. 2013
	4	N.C. Hills	25.18^0N-93.03^0E	15-30 Jan. 2013
	5	Kamrup	26.33^0N-91.25^0E	1-15 Feb. 2013
	6	Kokrajhar	26.40^0N-90.27^0E	15-28 Feb. 2013
	7	Sivasagar	26.59^0N-94.38^0E	1-15 Mar. 2013
	8	Tezpur	26.63^0N-92.8^0E	15-30 Mar. 2013

3.2. Isolation of Rhizobia

Individual nodules were dissected from the roots using a flame sterilized scalpel. The nodules were first washed with tap water to remove adherent soil. The nodules were then transferred to sterile petri dish and surface sterilized by immersion in 75% ethanol for 3 min and in 0.1% $HgCl_2$ for 5 min. The nodules were then washed 5–6 times with sterile distilled water and crushed by using the sterile glass rod. The white suspension obtained from the crushed nodule was then streaked on Yeast

Extract Mannitol Agar (YEMA) plate. The YEMA plates were incubated at 28±2°C for 3-5 days (Vincent, 1970). Single colonies of the isolates were picked up and re-streaked on YEMA plates for obtaining pure cultures.

3.3. Preparation and maintenance of pure culture

The pure cultures were maintained in two ways viz. in YEMA slant and in 50% glycerol stock.

A. YEMA slants were prepared by pouring YEMA media in test tubes. A colony of isolated bacterial sample is streaked in YEMA slants and kept in incubation for 3-5 days at 28±2°C. The slants were then kept in 4°C for temporary storage.

B. For preparation of glycerol stock the isolates were first inoculated in YEMA broth and incubated for 3-5 days at 28±2°C. After incubation 1.6 ml of bacterial broth is mixed with 0.4 ml of 50% glycerol in glass vials. The glass vials were then stored in -80^{0}C for permanent storage.

3.4. Reference strains

For screening and comparing the features of isolates, 4 reference strains *Bradyrhizobium japonicum* MTCC-120, *Rhizobium leguminosarum* MTCC-99, *Burkholderia cepacia* MTCC-4684 and *Mesorhizobium thiogangeticum* MTCC-7001 were obtained from Institute of Microbial Technology (IMTECH), Chandigarh in lyophilized form. Pure cultures of the reference strain were prepared for YEMA media and stored as per instruction.

3.5. Phenotypic characterization

3.5.1. Morphological characterization

Morphological features of the isolates were observed and compared with the morphological features of reference strains. For morphological characterization the isolates were cultured on YEMA plates and incubated at 28 ± 2^0C, after an incubation of 2-3 days individual colonies were observed for their size, color, shape and elevation (Vincent, 1970).

3.5.2. Microscopic characterization

Principle: Gram staining is a method of differentiating bacterial species into two groups, i.e. Gram negative and Gram positive. It is based on the chemical and physical properties of bacterial cell wall. The staining method depends on peptidoglycan which is present in a thick layer in Gram positive bacteria. A Gram positive reaction results in a purple/blue color while a Gram negative reaction results in a pink/red color.

Procedure: Microscopic slides of isolated and purified bacterial cultures were prepared for gram staining by the Vincent's method (Vincent, 1970). A drop of bacterial culture was taken and a thin smear was prepared on a glass slide. The smear was air-dried, heat fixed, stained with crystal violet for 1 minute and slightly washed with distilled water. The smear was then flooded with iodine solution for 1 minute and decolorized with 95% ethanol for 1 minute. The slide was then washed with distilled water and counterstained with safranin. The slide was then washed with distilled water, air dried and observed under a light microscope (Nikon, Japan) at 100x magnification using oil immersion.

3.6. Confirmatory tests

3.6.1. Growth on YEMA-Cr

Principle: Generally rhizobial colonies don't absorb congo red dye and appeared as a white color colony and all other contaminating bacteria absorb the dye and appeared as a red color colony on YEMA-Cr media (Somasegaran & Hoben, 1994).

Procedure: The isolates were streaked on YEMA media containing 2.5ml/l congo red solution and incubated at 28 ± 2^0C for 3-5 days in the dark until growth appeared. Isolates with white color colonies were considered as rhizobia.

3.6.2. Ketolactose test

Principle: This test is done in order to distinguish rhizobia from *Agrobacterium*. *Agrobacterium* utilizes lactose whereas rhizobia don't. The enzyme ketolactose convert lactose to ketolactose. This production of ketolactose is detected by adding Benedict's reagent. If the lactose is being utilized, a yellow ring is formed around the colony whereas no yellow ring formation takes place if lactose is not being utilized (Sharma *et al*., 2010).

Procedure: The isolates were streaked on ketolactose agar media and incubated at 28± 2^0C for 3-5 days. The plates are then overlaid with Benedict's reagent. If the isolate belongs to *Agrobacterium* a yellow coloration will be observed surrounding the streaked areas while the rhizobial colonies show no such coloration.

3.7. Biochemical characterization

The isolates were screened for their biochemical features by following standard procedure.The experiments were carried out in triplicate. The following biochemical tests were performed with the isolates.

3.7.1. Catalase test

Principle: Most of the microorganisms have the characteristic of showing catalase activity. These organisms utilize O_2 to produce H_2O_2. The H_2O_2 is toxic to their enzyme system. Hence, these organisms produce an enzyme called catalase, which mediates the breakdown of H_2O_2 into O_2 and H_2O. The catalase enzyme neutralizes the bactericidal effects of H_2O_2 and protects them.

Procedure: A single colony from a pure culture plate was picked using a sterile loop and mixed with 3% hydrogen peroxide on a glass slide. Appearance of bubbles within few seconds indicates production of catalase enzyme by the isolate (Kumari *et al*., 2010). The lack of catalase is evident by a lack of or weak bubble production.

3.7.2. Oxidase test

Principle: Generally oxidase test is coducted to determine the ability of bacteria to produce cytochrome c oxidase, an enzyme involved in bacterial electron transport chain. When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to the blue color end product. When the enzyme is not present, the reagent remains reduced and is colorless.

Procedure: A single colony from a pure culture plate was picked using a sterile loop and smeared on oxidase discs. Blue coloration of the discs within few seconds indicates positive oxidase activity (Kovacs, 1956). No change in color indicates absence of oxidase enzyme.

3.7.3. Nitrate reduction test

Principle: Nitrate reduction test differentiates microbes on the basis of their ability to produce the nitrate reductase enzyme**.** The nitrate reductase enzyme hydrolyzes nitrate to nitrite, which may then again be degraded to various nitrogen products like nitrogen oxide, nitrous oxide and ammonia. If the organism has reduced

nitrate to nitrite the medium will turn blue after the addition of the nitrate reagents. If the medium does not turn red after the addition of the reagents, add a small amount of powdered zinc. If the broth medium turns red after the addition of the zinc, it indicates that unreduced nitrate was present. Therefore, a red color on the second step is a negative result.

Procedure: A single colony from a pure culture plate was picked using a sterile loop and inoculated in nitrate broth, incubated for 3 days at 28 ± 2^0C. After incubation few drops of dilute sulfuric acid and few drops of nitrate test reagent were added to the broth. Development of the blue color was considered as a positive nitrate reduction activity (Kumari *et al*., 2010).

3.7.4. Starch hydrolysis test

Principle: Starch is an insoluble polymer of glucose, which acts as a source of carbon for microorganisms. This test is carried out to determine the ability of bacteria to use starch as a source of carbon and energy for growth. Use of starch is accomplished by an enzyme called alpha-amylase. After inoculation and overnight incubation, iodine reagent is added to detect the presence of starch.

Procedure: In this test the isolates were streaked on starch agar plates and incubated for 2-3 days at 28± 2^0C. After incubation the plates were flooded with iodine solution and observe for starch hydrolysis around the colonies (Singh *et al*., 2008). Iodine solution reacts with starch and forms a blue-black color in the culture medium. Clear halos surrounding colonies are indicative of their ability to digest the starch in the medium due to the presence of alpha-amylase.

3.7.5. Urease test

Principle: Urease test is used to determine the ability of bacterial isolates to produce urease enzyme which splits urea to ammonia and carbon dioxide. The

ammonia combines with carbon dioxide and water to form ammonium carbonate, which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.

Procedure: A single colony from a pure culture plate was picked using a sterile loop and inoculated in urea broth containing phenol red as a pH indicator incubated for 3 days at 28 ± 2^0C. Change in color of medium from red to pink can be taken as a positive test for urease production (Kumari *et al*., 2010).

3.7.6. Citrate test

Principle: Citrate test or citrate utilization test is carried out to determine the ability of isolates to utilize sodium citrate as its only carbon source. When citrate is used as a carbon and energy source, alkaline carbonates and bicarbonates are produced ultimately. Growth usually results in the bromothymol blue indicator, turning from green to blue.

Procedure: Citrate test was performed by streaking the isolates on a Simmons citrate agar slant. The tubes were then incubated at 28 ± 2^0C for 3 days. Change of media color to blue indicates the utilization of citrate (Kumari *et al*., 2010).

3.7.7. Gelatin hydrolysis test

Principle: This test is carried out to determine the ability of isolates to produce gelatinase enzyme that liquefy or hydrolyses gelatin. Hydrolysis of gelatin indicate the presence of gelatinase enzyme. This process takes place in 2 sequential reactions. In the first reaction, gelatinase degrade gelatin to polypeptides. Then, the polypeptides are further converted into amino acids.

Procedure: A single colony from a pure culture plate was picked using a sterile loop and streaked on nutrient gelatin medium and incubated for 2-3 days at 28 ± 2^oC. On subjecting the isolates to low temperature treatment at 4°C for 30 min, the cultures which

produce gelatinase remains liquefied while others, due to presence of gelatin becomes solid (Kumari *et al*., 2010).

3.8. Plant growth promoting ability test

3.8.1. Indole acetic acid (IAA) production

Principle: The aim of this test is to determine the ability of isolates to produce IAA. Indole acetic acid is one of the most available and physiologically active auxins. It is also a known fact that IAA is a common product of L- tryptophan metabolism produced by several microorganisms.

Procedure: To observe IAA production, isolates were incubated on YEM broth medium supplemented with L-tryptophan (0.1%) for 24 h. Supernatant of the isolates were collected and transferred separately to a fresh tube to which 100 µl of 10 mM O-phosphoric acid and 4 ml of Salkowski reagent were added. Mixtures were incubated at room temperature for 25 min and the production of IAA was observed in a spectrophotometer by measuring the O.D. at 540 nm (Bric *et al*., 1991). The amount of IAA production was determined by comparing the data with a standard graph prepared by measuring the O.D. with different concentration (10 µg/ml -100 µg/ml) of IAA (Gordon & Weber, 1951).

3.8.2. Phosphate solubilisation

Principle: This test is used to determine the phosphate solubilization ability of the isolates. The phosphate solubilization ability of microorganisms is believed to be one of the most important plan growth promoting traits associated with plants. It is generally accepted that the mechanism of phosphate solubilization by bacteria is associated with the release of low molecular weight organic acids, through which their hydroxyl and carboxyl groups chelate the cations bound to phosphate and thus solubilizes phosphate.

$$PSE = \frac{\text{Solubilization diameter (mm)} - \text{Colony diameter (mm)}}{\text{Colony diameter (mm)}} \times 100$$

Procedure: Thephosphate solubilization ability of the isolates was determined by spotting the isolates on Pikovskya's agar plates. Plates were then incubated at 28 ± 2°C for 3 days and observed for the formation of the halo zone around the colonies (Pikovskaya, 1948). The ability of the isolates was expressed in terms of Phosphate Solubilization Efficiency or PSE.

3.8.3. Ammonia production

Principle: This test is used to determine the ability of microorganisms to produce ammonia by utilizing Nessler's reagent.

Procedure: Overnight grown bacterial cultures were inoculated in 10 ml peptone broth and incubated at 28 ± 2°C for 2-3 days. After incubation, 0.5 ml of Nessler's reagent was added. The development of faint yellow to dark brown color indicates the production of ammonia (Kumar *et al*., 2012).

3.9. Acidic pH and salt tolerance test

3.9.1. Acidic pH tolerance test

The ability of the isolates to tolerate acidic pH was determined by streaking the isolates on 3 different YEMA plates adjusted with 3 different pH (4.0, 5.0 and 6.0). The plates were then incubated at 28 ± 2°C for 2-3 days and observed for their ability to grow (Kucuk *et al*., 2006). Growth of isolates on YEMA media adjusted at pH-7.0 was used as a control.

3.9.2. Salt tolerance test

The ability of the isolates to grow in high salt concentration was also tested on YEMA plates. The isolates were streaked and incubated on 3 different YEMA plates adjusted with 3 different concentrations (1.0%, 2.0% and 3.0%) of NaCl. The isolates

were then observed for their ability to grow after an incubation at 28 ± 2°C for 2-3 days (Romdhane *et al*., 2006). Growth of isolates on YEMA media adjusted at 0.1% NaCl was used as a control.

3.10. DNA extraction

Principle: Extraction of DNA is required for determining the phylogenetic identity of the isolated strains and for determining the presence of symbiotic genes. Genomic DNA extraction of selected isolates and reference strains was performed using the standard Phenol-chloroform extraction procedure (Sambrook *et al*., 1989).

Procedure: All the isolates were inoculated in YEM broth and incubated for 24 hr at 28 ^{0}C. By centrifugation supernatant was discarded and to the pellet 467 µl TE buffer, 30 µl of 10% SDS and 3 µl of proteinase K (20 mg/ml) was added and incubated at 55 °C for 30 min. After incubation an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added, centrifuged and to the aqueous layer an equal volume of chloroform/isoamyl alcohol (24:1) was added and again centrifuged. The aqueous phase was transferred to a fresh tube and double volume of 95% ethanol was added and incubated at -20 °C for overnight. The precipitated DNA was washed with 70% alcohol, air dried and finally dissolved in 100 µl of nuclease free water.

3.11. Visualization of DNA bands

For visualizing the extracted DNA 1% agarose gel was prepared. For preparing 1% agarose gel, 1gm of agarose was dissolved in 100 ml of autoclaved distilled water. Agarose was completely dissolved by heating in a microwave oven. The gel was then allowed to cool by keeping it at room temperature and to it 3 µl of ethidium bromide was added. The gel was then poured into a gel casting tray and allowed to solidify. The solidified gel was then placed in an electrophoresis tank and

submerged with 1x TAE. 5 µl of DNA was mixed with 2.5 µl of 6 X gel loading dye and loaded into the wells of the gel. Along with the extracted DNA samples 1 kb DNA ladder was loaded into the wells and used as marker. The electrophoresis was carried out at 55 volts for 50 min and the DNA band was visualized by using a gel documentation system (BioRad).

3.12. PCR amplification of *nifH*gene

PCR amplification of *nifH* gene of isolates and reference strains was carried out to determine the presence of *nifH* gene (Zehr & McReynolds, 1989). Amplification reaction was carried out with a total volume of 50 µl containing genomic DNA (30 ng), dNTP's (2.5 mM), primers (10 pmol), PCR buffer (10 x), Taq polymerase (1.5 U) and nuclease free water. Primers used for *nifH* gene amplification is given in Table 2. The reaction condition used in *nifH* gene amplification is given in Table 3.

Table 2. *nifH* gene primers

Primer name	Sequence
zehrf (Forward)	5'TGCGACCCAAAAGCAGA3'
zehr (Reverse)	5'AAAGCCATCATCTCACC3'

Table 3. Reaction condition for *nifH* gene amplification

PCR steps	Temperature	Time
Initial denaturation	95°C	5 min
Denaturation (30 cycles)	94 ^{o}C	30 sec
Annealing (30 cycles)	58 ^{o}C	30 sec
Extension (30 cycles)	72 ^{o}C	45 sec
Final extension	72 ^{o}C	7 min

3.13. Visualization of amplified *nifH* gene bands

For visualizing the amplified *nifH* gene bands 1.5% agarose gel was prepared. For preparing 1.5% agarose gel 1.5 gm of agarose was dissolved in 100 ml of autoclaved distilled water. Agarose was completely dissolved by heating in a microwave oven. The gel was then allowed to cool by keeping it at room temperature and to it 3 µl of ethidium bromide was added. The gel was then poured into a gel casting tray and allowed to solidify. The solidified gel was then placed in an electrophoresis tank and submerged with 1x TAE. 5 µl of DNA was mixed with 2.5 µl of 6 X gel loading dye and loaded into the wells of the gel. For determining the size of the amplified *nifH* gene a 100 bp DNA ladder was loaded into the wells and used as marker. The electrophoresis was carried out at 55 volts for 50 min and the DNA band was visualized by using gel documentation system.

3.14. PCR amplification of *nodC* gene

PCR amplification of *nodC* gene of selected isolates and reference strains was carried out to determine the presence of *nodC* gene (Dubey *et al*., 2010).The amplification reaction was carried out with a total volume of 50 µl containing genomic DNA (30 ng), dNTP's (2.5 mM), primers (10 pmol), PCR buffer (10 x), Taq polymerase (1.5 U) and nuclease free water. Primers used for *nodC* gene amplification is given in Table 4. The reaction condition used in *nodC* gene amplification is given in Table 5.

Table 4. *nodC* gene primers

Primer name	**Sequence**
nodC1 (Forward)	5'GCCATAGTGGCAACCGTCGT3'
nodC2 (Reverse)	5'TCACTCGCCGCTGCAAGTC3'

Table 5. Reaction condition for *nodC* gene amplification

PCR steps	Temperature	Time
Initial denaturation	95°C	5 min
Denaturation (30 cycles)	94 ^{o}C	30 sec
Annealing (30 cycles)	56 ^{o}C	30 sec
Extension (30 cycles)	72 ^{o}C	45 sec
Final extension	72 ^{o}C	7 min

3.15. Visualization of amplified *nodC* gene bands

For visualizing the amplified *nodC* gene bands 1.5% agarose gel was prepared. For preparing 1.5% agarose gel 1.5 gm of agarose was dissolved in 100 ml of autoclaved distilled water. Agarose was completely dissolved by heating in a microwave oven. The gel was then allowed to cool by keeping it at room temperature and to it 3 µl of ethidium bromide was added. The gel was then poured into a gel casting tray and allowed to solidify. The solidified gel was then placed in an electrophoresis tank and submerged with 1x TAE. 5 µl of DNA was mixed with 2.5 µl of 6 X gel loading dye and loaded into the wells of the gel. For determining the size of the amplified *nodC* gene a 100 bp DNA ladder was loaded into the wells and used as marker. The electrophoresis was carried out at 55 volts for 50 min and the DNA band was visualized by using gel documentation system.

3.16. PCR amplification of 16S rDNA gene

PCR amplification of 16S rDNA gene of the isolates and reference strains was carried out by using universal primers (Weisburg et al. 1991). The amplification reaction was carried out with a total volume of 50 µl containing genomic DNA (30 ng), dNTP's (2.5 mM), primers (10 pmol), PCR buffer (10 x), Taq polymerase (1.5 U) and nuclease free water. Primers used for amplification is given in Table 6. The reaction condition used in 16S rDNA gene amplification is given in Table 7.

Table 6. 16S rDNA gene primers

Primer name	Sequence
27f (Forward)	5'GCCATAGTGGCAACCGTCGT3'
1492r (Reverse)	5'TCACTCGCCGCTGCAAGTC3'

Table 7. Reaction condition for 16S rDNA gene amplification

PCR steps	Temperature	Time
Initial denaturation	95°C	5 min
Denaturation } (30 cycles)	94 ^{o}C	1 min
Annealing } (30 cycles)	55 ^{o}C	1 min
Extension } (30 cycles)	72 ^{o}C	2 min
Final extension	72 ^{o}C	7 min

3.17. Visualization of amplified 16S rDNA gene bands

For visualizing the amplified 16S rDNA gene bands 1.5% agarose gel was prepared. For preparing 1.5% agarose gel 1.5 gm of agarose was dissolved in 100 ml of autoclaved distilled water. Agarose was completely dissolved by heating in a microwave oven. The gel was then allowed to cool by keeping it at room temperature and to it 3 µl of ethidium bromide was added. The gel was then poured into a gel casting tray and allowed to solidify. The solidified gel was then placed in an electrophoresis tank and submerged with 1x TAE. 5 µl of DNA was mixed with 2.5 µl of 6 X gel loading dye and loaded into the wells of the gel. A 100 bp DNA ladder was loaded into the wells and used as marker. The electrophoresis was carried out at 55 volts for 50 min and the DNA band was visualized by using gel documentation system.

3.18. RFLP analysis of 16S rDNA gene

Principle: Restriction Fragment Length Polymorphism or RFLP is a widely used technique for genetic diversity analysis. In the present study PCR-RFLP analysis of 16S rDNA genewas carried out for determining their genetic diversity (Laguerre *et al*., 1994).

Procedure: PCR amplified products of isolates and reference strains were digested with restriction enzymes (*AluI*, *HinfI*). Restriction digestions were performed in 15 µl reaction volume containing 10 µl of PCR amplified product, 2 µl of restriction enzyme and 3 µl buffer. The reaction mixture was incubated at 37°C for overnight.

3.18.1. Visualization of digested PCR-RFLP bands

The RFLP patterns obtained from the digestion were visualized after electrophoresis on 2% agarose gel. For preparing 2% agarose gel 2gm of agarose was dissolved in 100 ml of autoclaved distilled water. Agarose was completely dissolved by heating in a microwave oven. The gel was then allowed to cool by keeping it at room temperature and to it 3 µl of ethidium bromide was added. The gel was then poured into a gel casting tray and allowed to solidify. The solidified gel was then placed in an electrophoresis tank and submerged with 1x TAE. 5 µl of DNA was mixed with 2.5 µl of 6 X gel loading dye and loaded into the wells of the gel. For determining the size of the amplified 16S rDNA genea 100 bp DNA ladder was loaded into the wells and used as marker. The electrophoresis was carried out at 55 volts for 50 min and the DNA band was visualized by using gel documentation system.

3.18.2 Construction of RFLP dendrogram

A simple matching coefficient was calculated to construct a similarity matrix and the UPGMA (Unweighted Pair Grouping with Mathematic Average) algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram by using NTSYS-pc package (Rohlf, 1998).

3.19. 16S rDNA sequencing and analysis

The PCR amplified products of 16S rDNA genes of the isolates were purified and sequenced by using automated DNA Sequencer (ABI 3500 Genetic Analyzer; Applied Biosystem) at Genomics and DNA Bar-coding Lab, Department of Biotechnology, Assam University. The sequencing reaction was performed with a total volume of 20 µl containing BDTv3.1 Ready reaction mix (2.5 x), sequencing buffer (5 x), primers (3.2 pmol), DNA (20 ng) and nuclease free water. The chain termination reaction was carried out by Thermal cycler and fragments were purified by Sodium Acetate/EDTA/Ethanol method prior to run in the 3500 Genetic analyzer.

Table 8. Reaction condition for 16S rDNA sequencing

PCR steps		Temperature	Time
Initial denaturation		96°C	1 min
Denaturation	(25 cycles)	96 ^{o}C	10 sec
Annealing	(25 cycles)	50 ^{o}C	5 sec
Extension	(25 cycles)	60 ^{o}C	4 min

The quality of the sequences was assessed and edited using BioEdit program (Hall, 1999). NCBI-BLAST analysis was carried out with the 16S rDNA sequences of the isolates to determine their closest relatives and to predict their identity.

3.20. Phylogenetic tree construction

The acquired sequences from the present study together with the related sequences obtained from GenBank with the NCBI-BLAST program, were aligned

using the CLUSTAL W program (Thompson *et al*., 1994). Phylogenetic trees of the sequenced isolates were constructed with the MEGA 4.0 software package using the neighbor-joining method (Tamura *et al*., 2007).

3.21. Assessment of nodulation and plant growth

Seeds of selected plantswere surface sterilized by gently shaking it in 70% ethanol for 2 min and $HgCl_2$ for 3 min. The bacterial isolates were grown in YEM broth medium at 28°C for 3-5 days and inoculated on the emerging seedlings of their original hosts (Amel *et al*., 2013). Seeds inoculated with bacteria isolate were sown in plastic pot containing vermiculite-quartz sand mixture. The sand mixture was moistened with nitrogen free nutrient solution at regular time intervals. The treatment was carried out in triplicate (three plants per pot) and nine plants were considered for each isolate. Un-inoculated plants were kept as control. The plants were harvested after 30-45 days of planting and their vegetative parameters were measured. The experiment was performed in a completely randomized design with three replications. The parameters evaluated were presence and absence of nodules, shoot and root height, shoot and root dry weight. Statistical Analysis of Variance (ANOVA) was carried out to identify significant difference in each vegetative parameter between treated and control at 5% significance level by using SPSS 16.0 software.