

Original Research Article

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Characterization of Diazotrophs Isolated from Sugarcane Rhizosphere in Different Regions of Bangladesh

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ABSTRACT

The experiment was conducted at the Soil Microbiology Laboratory of the Department of Soil Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur during the period from January, 2011 to June, 2011 with a view to isolate and characterized promising diazotrophs from sugarcane rhizosphere. Twenty four bacterial strains were isolated from sugarcane rhizosphere soils. Among the 24 isolated strains thirteen of them were found as Gram negative and eleven were Gram positive. Seventeen of the strains were positive for cellulose activity. Fifteen of the strains were found as positive for phosphate solubilisation activity. All of these bacterial strains were evaluated for nitrogen fixation activity, among them 21 isolates were found as ARA positive. The highest ARA was found in strain *Acinetobacter calcoaceticus* (BUSo 9) ($3.74 \times 10^{-4} \mu\text{mol C}_2\text{H}_4 \text{ cfu}^{-1}\text{hr}^{-1}$) which was statistically similar to *Bacillus cereus* (BUSo 13) ($1.54 \times 10^{-4} \mu\text{mol C}_2\text{H}_4 \text{ cfu}^{-1}\text{hr}^{-1}$). All of the strains were found as IAA producing. Two promising strains were selected for 16SrDNA sequencing. From 16S rDNA sequence analysis of the strains it was observed that ninety nine per cent sequence identity was observed between the 16S rDNA sequence of BUSo 9 and *Acinetobacter calcoaceticus* and also ninety nine per cent sequence identity was observed between the 16S rDNA sequence of BUSo 13 and *Bacillus cereus*.

Keywords

Characterization,
Diazotrophs,
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Introduction

Biological nitrogen fixation (BNF) by diazotrophic bacteria is an important process when N is a limiting factor for the growth of organisms. Diazotrophic bacteria are found in symbiosis with plants forming root nodule

structures, or as free-living bacteria in the rhizosphere or as associative bacteria in a large variety of plant species, mainly monocotyledons such as rice (*Oryza sativa* L.) (Döbereiner and Pedrosa, 1987; Khammas *et al.*, 1989; Gillis *et al.*, 1995; Xie and Yokota, 2005), sugarcane (*Saccharum*

officinarum L.) (Döbereiner and Ruschel, 1958; Reis *et al.*, 1994; Baldani *et al.*, 1997), corn (*Zea mays* L.) (Döbereiner and Ruschel, 1958; Magalhães *et al.*, 1979; Reis *et al.*, 2004), and wheat (*Triticum aestivum*, L.) (Neal and Larson, 1976). Non-symbiotic (associative or free living) diazotrophic bacteria also promote plant growth by BNF and through the production of metabolites that stimulate root growth. High rates of nitrogen (N) fertilizer are often applied to maximize sugarcane yields. High cost for N fertilizers and off-site N losses are a problem, as are declining sugarcane yields despite high agronomic input (Bell *et al.*, 2007; Brodie *et al.*, 2008). In addition, manufacture of synthetic N fertilizer uses $\approx 2\%$ of global energy consumption. One avenue to remediate the problem associated with synthetic N fertilizers is the use of microbes capable of biological N_2 fixation (BNF) (de Carvalho *et al.*, 2011). There is evidence that endophytic bacteria able to fix N_2 contribute to the N budget of Brazilian sugarcane (Boddey *et al.*, 2003).

The efficacy of diazotrophic bacteria varies considerably in sugarcane crops (Yoneyama *et al.*, 1997). Further, rhizosphere bacteria including diazotrophic bacteria have cultivar-specific relationships, and sugarcane varieties may have specific controls aimed at attracting specific diazotrophs (de Carvalho *et al.*, 2011). Therefore, it is important to isolate and characterized highly N_2 fixing and growth promoting diazotrophs from sugarcane rhizosphere in Bangladesh and use of these diazotrophs for making biofertilizers for commercial and environmentally safe cultivation of sugarcane. But no research works on diazotrophs related to sugarcane in Bangladesh has been done yet. Keeping all this views in mind the present study was undertaken to isolate and characterization diazotrophs from sugarcane rhizosphere on the basis of N_2 -fixation, phytohormone

production, physiological and biochemical properties.

Materials and Methods

The experiment was conducted at the Soil Microbiology Laboratory of the Department of Soil Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur during the period from January, 2011 to June, 2011 with a view to isolate and characterized promising diazotrophs from sugarcane rhizosphere.

Sampling procedures for isolation of diazotroph

Rhizosphere soil samples were collected from six locations comprising six Agro Ecological Zones (AEZ) of Bangladesh viz. Ishurdi (AEZ-11), Thakurgaon (AEZ-1), Joypurhat (AEZ-3), Gazipur (AEZ-28), Habiganj (AEZ-29) and Jamalpur (AEZ-9). The samples were collected randomly from three-month aged sugarcane field. Samples of sugarcane root and root adhering soil (rhizosphere soil) were collected randomly from three sugarcane variety i.e. Isd 37, Isd 39 and Isd 40 for isolation of diazotrophs. For each region samples were collected from three locations. A composite sample were taken to the laboratory in an ice box and kept at 4°C temperature before isolation.

Isolation of diazotroph from sugarcane rhizosphere

Isolation of different diazotrophic bacteria from sugarcane rhizosphere was done in different semisolid media i.e Nfb media and Burks media. Composition of the Nfb medium was: 5 g malic acid, 0.5 g K_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g NaCl and 0.5% bromothymol blue in 0.2 N KOH (2 ml), 1.64 % Fe-EDTA solution (4 ml), 2 g agar L^{-1} for semi solid medium and 15 g agar L^{-1} for solid

medium. The pH of the medium was adjusted with KOH to 7.20. Composition of Burks medium was: 20 g sucrose, 0.80 g K₂HPO₄, 0.20 g KH₂PO₄, 0.13 g CaSO₄, 0.20 g MgSO₄ 7H₂O, 0.000253 g Na₂MoO₄.2H₂O, 0.00145 g FeCl₃.2H₂O, 5.0 ml bromothymol Blue (0.5% in ethanol), 2 g agar L⁻¹ for semi solid medium and 15 g agar L⁻¹ for solid medium. The pH of the medium was adjusted with NaOH to 7.00. Approximately 10 g of sugarcane root with adhering soil was placed in to 250 mL Erlenmeyer flask containing 95 mL sterilized water, and content was shaken for 15 to 20 min. A series of 10-fold dilutions were prepared up to 10⁻⁸ of rhizosphere soil. Sterilized Nfb and Burks semisolid medium were used for isolation of diazotrophs. Inoculation was done in test tubes containing 10 mL of semisolid media. From the lower dilutions (10⁻³ to 10⁻⁷) 0.1 mL was inoculated in each test tube. The mouth of test tube was closed with cotton plug and placed in an incubator adjusted to 28°C for 2 days. For purification of colonies from the pellicle formed in semisolid media bacterial cells were streaked out with a sterilized needle and inoculated again in the same semisolid media. This process was carried out five times. Then the diazotroph bacterial culture was grown in respective solid media for obtaining single colonies. From the single colonies morphological characteristics were studied and depending on different colony characters specific names of the isolates were given. These isolates were preserved in slant of specific medium with addition of glycerol and oil emulsion. All isolates were also preserved in eppendorf tube containing 1 mL 20% glycerol solution and incubated at -20°C prior to characterization. Each isolate was preserved in several numbers of slants and eppendorf tube for further use.

Characterization of diazotrophic isolates:

A preliminary experiment was conducted in the Soil Microbiology laboratory of the

Bangabandhu Sheikh Mujibur Rahman Agricultural University to characterize all of the isolates. Biochemical characteristics of different diazotrophic isolates were determined on the following aspects: Gram reaction, KOH test, acetylene reduction assay, IAA production, and cellulose degrading activity and phosphate solubilizing assay.

Gram staining and KOH test

About 48h of pure bacterial isolates were tested for Gram staining and KOH test. Gram staining slides were prepared by heat fixed smear of bacteria flooded with two drops of crystal violet for one minute. The crystal violet was then gently washed off with sterilized distilled water. The smear was sealed with iodine for one minute followed by washing with water. Using a Pasteur pipette alcohol solution was gently dropped to remove the stain. After washed with water the smear was counter stained with safranin for 30 sec. The slides were washed with sterilized distilled water and air dried. The stained smear was observed under the light microscope using oil emulsion at 10x to 100x magnification.

A Gram-positive strain appeared bluish or purple and Gram-negative strain was reddish in colour (Naher *et al.*, 2009). For KOH test, pure culture isolates from media plates were dropped on slides and one drop of freshly prepared 3.0 % KOH was added and mixed with culture with the help of sterile needle. The Gram-negative bacteria suspension becomes thick and sticky while Gram positive remain the same (Naher *et al.*, 2009).

Determination of cellulase activity

The cellulase activity was determined according to James *et al.*, (2002). The bacterial culture was grown in Jensen's broth and incubated for 48 hours. Cellulose degradation was carried out on Jensen's agar

plates with 0.1% carboxymethyl cellulose (CMC). Plates were spot inoculated with 10 µl liquid culture of the strains. After 24 hours of incubation period the colonies were streaked and washed off with sterile water and discarded. The plates were stained with 0.1 % Congo red solution for 30 min. After stained, plates were rinsed with 1 M NaCl. The cellulose degrading enzyme activated plates showed clear halo zone. The halo-zones indicated positive in reaction.

Phosphate solubilizing assay

Test for P-solubilisation was done following Sharma *et al.*, (2012). The plates were prepared with Pikovaskya's medium. The cultures of diazotroph isolates were spot inoculated on the plates and incubated in an incubator at 28°C for 3- 5 days. Formation of clear zone around the microbial colonies indicated phosphate solubilization. Composition of Pikovskaya media in 1 litre: Glucose-10 g, Ca₃(PO₄)₂-5g, (NH₄)₂SO₄-0.5 g, NaCl-0.2 g, MgSO₄.7H₂O-0.1 g, KCL-0.2 g, Yeast extract-0.5 g, MnSO₄.H₂O-0.002 g, FeSO₄.7H₂O-0.002 g and Agar-15 g (Pikovskaya, 1948).

Estimation of nitrogenase enzyme activity (acetylene reduction assay)

A 1.0 mL of the culture was transferred into an air-tight 30 ml bottle containing 10 ml of Nfb semi-solid medium. After the pellicle formation (72 h), the bottles were injected with 5% (v/v) acetylene gas with simultaneous removal of the same volume of air. The bottles then incubated at 30°C for 24h. 1 ml of gas was withdrawn and transferred to 7 ml VacutainerTM tubes. The ethylene gas produced was measured using G-300 gas chromatograph (AmericanTM) equipped with an FID and 1-m Porapak N column (Naher *et al.*, 2009).

IAA production

Both *Rhizobium* and diazotrophic isolates were inoculated in Jensen's broth with addition of tryptophan (2 mg mL⁻¹) and incubated at 28 ± 2⁰C for 48 h. The culture was then centrifuged at 7000 rpm for 7 min and 1 mL of the supernatant was mixed with 2 ml of Salkowsky's reagent. The IAA concentration was determined using spectrophotometer at 535 nm (Naher *et al.*, 2009).

DNA extraction

Isolation of genomic DNA

For genomic DNA isolation, selected bacterial isolates were cultured in nutrient broth at 27°C and 120 rpm in a shaker incubator. About 24 hours old cultures were subjected for genomic DNA isolation. A commercial DNA extraction kit (manufactured by ATP Genomic DNA Mini Kit, ATP Biotech Inc.) was used and the genomic DNA was extracted following the instructions of the manufacturer. The polymerase chain reaction (PCR) for amplification of the targeted 16SrDNA of the two-representative presumptive isolates BUSo 9 and BUSo13 was performed with the universal primer sets 24 F (forward primer) and 1540 R (reverse primer). For amplification of soil bacterial isolates, *Streptomyces* genus specific primer set Streptom F and Streptom R was used. All primers (Sigma Ltd.) were preserved in the Biotechnology Laboratory, BSMRAU, Gazipur which have been routinely used for molecular identification of fish pathogenic and soil bacteria. Sequences of these primers are given in Table 1.

The PCR amplification was performed in a PCR Thermocycler (Eppendorf Ltd). The PCR product was purified by using a

commercial Gel/PCR Purification Kit (FavorPrep™, Favorgen® Biotech Corp) following the manufacturer's protocol.

DNA sequencing

The purified PCR products of the soil bacteria and potential antimicrobial agent producing soil bacterial isolates along with sequencing primer were sent to the laboratory of Centre for Advanced Research in Sciences (CARS), Dhaka University for 16sRDNA sequencing.

Phylogenetic analysis

DNA sequences (FASTA format) of the isolates were then used for DNA sequence homology study and phylogenetic analysis using the BLAST and Phylogeny.fr web based soft ware.

Results and Discussion

Gram staining and KOH test

The diazotroph isolated from different sugarcane growing regions were tested for Gram reaction and KOH test. Among the 24 isolated strains 13 of them were found as Gram negative and 11 were Gram positive. The KOH test showed that 13 of the strains were positive and 11 were negative in KOH reaction (Table 2).

Cellulase activity

Jensen-CMC plate assayed to determine cellulose activity. The plates spotted with diazotrophic broth showed a clear halo zone after stained with 0.1% Congo red indicating cellulose activity. Seventeen of the rhizosphere isolates were positive for cellulose activity (Plate 1).

Phosphate solubilizing activity

Pikovskaya plates were assayed to determine

Phosphate solubilizing activity (Plate 2). The cultures of diazotroph isolates were spot inoculated on the plates and incubated in an incubator at 28°C for 3- 5 days.

Fifteen of the diazotroph isolates were found as positive for phosphate solubilization activity.

Acetylene reduction assay (ARA)

Twenty-four bacterial strains were evaluated for nitrogen fixation activity (Table 2). The ARA value ranges from 5.16×10^{-10} to 3.74×10^{-4} $\mu\text{mol C}_2\text{H}_4 \text{ cfu}^{-1}\text{hr}^{-1}$. The highest ARA was found in isolate BUSo 9 (3.74×10^{-4} $\mu\text{mol C}_2\text{H}_4 \text{ cfu}^{-1}\text{hr}^{-1}$) which was statistically at par with BUSo 13 (1.54×10^{-4} $\mu\text{mol C}_2\text{H}_4 \text{ cfu}^{-1}\text{hr}^{-1}$) and the lowest was obtained from BUSo 24 (5.16×10^{-10} $\mu\text{mol C}_2\text{H}_4 \text{ cfu}^{-1}\text{hr}^{-1}$). Several isolates were high in ARA as observed for BUSo 3, BUSo 19, BUSo 20 and BUSo 6. Many researchers reported that diazotrophic bacteria have been linked with the high nitrogen fixation reported particularly in sugarcane where these bacteria were found in high numbers (Boddey *et al.*, 1991, 1995 a, b; Döbereiner *et al.*, 1995 a, b; James and Olivares, 1997).

Indoleacetic acid (IAA) production

Isolated diazotroph were able to produce high amount of IAA (Fig.1). Appearance of red colour indicating IAA production by Diazotrophs (Plate 1). IAA production of isolates differed significantly among strains. In the presence of tryptophan the bacterial isolates produced higher IAA which varied from 30 to 180 $\mu\text{g mL}^{-1}$. The highest IAA was recorded in strain BUSo 5 (180 $\mu\text{g mL}^{-1}$) followed by strain BUSo 3 and BUSo 22 (both 150 $\mu\text{g mL}^{-1}$). This finding was at par with that of Keyeo *et al.*, (2011) reported rice diazotrophs produces phytohormone indole-3-acetic acid (IAA) in different concentration.

Identification of diazotrophs

Quantification of bacterial genomic DNA of *Acinetobacter calcoaceticus* (BUSo 9) and *Bacillus cereus* (BUSo 13) compare with 1 kb

DNA ladder (AccuLadder, BIONEER) as a Marker (L1) was shown in Figure 2. PCR amplification of *Acinetobacter sp.* Isolate (BUSo 9) and *Bacillus sp.* Isolate (BUSo 13) was shown in Figure 3.

Table.1 Primer sequences used for PCR amplification (Primer pair Sequence (5'-3') and Expected PCR amplicon size (bp)

Primer pair	Sequence (5'-3')	Expected PCR amplicon size (bp)
24 F 1540R	AGAGTTTGATCCTGGCT AAGGAGGTGATCCTGGCT	1516

Table.2 Biochemical characteristics of isolates obtained from rhizosphere soils

Isolates	Gram reaction	KOH reaction	Cellulose degradation	P solubilization	ARA ($\mu\text{mol C}_2\text{H}_4^{-1}\text{cfu}^{-1}\text{h}^{-1}$)
BUSo1	+	-	-	-	Nd*
BUSo2	+	-	-	-	Nd*
BUSo 3	-	+	+	+	8.56×10^{-5} b
BUSo 4	+	-	-	+	7.44×10^{-7} f
BUSo 5	-	+	+	-	7.99×10^{-10} f
BUSo 6	-	+	-	+	1.36×10^{-7} cd
BUSo 7	+	-	+	+	5.48×10^{-9} f
BUSo 8	-	+	+	+	8.61×10^{-10} hi
BUSo 9	-	+	+	+	3.74×10^{-4} a
BUSo10	+	-	+	-	3.26×10^{-9} fg
BUSo11	-	+	+	+	9.38×10^{-10} hi
BUSo12	-	+	+	-	14.89×10^{-10} gh
BUSo13	+	-	+	+	1.54×10^{-4} ab
BUSo14	+	-	+	+	8.97×10^{-9} f
BUSo15	+	-	-	+	5.44×10^{-9} f
BUSo 16	-	+	-	-	Nd*
BUSo 17	-	+	+	+	5.65×10^{-8} de
BUSo 18	+	-	+	-	8.46×10^{-8} cde
BUSo 19	-	+	+	+	2.11×10^{-7} c
BUSo 20	+	-	-	+	2.26×10^{-7} c
BUSo 21	+	-	+	+	3.94×10^{-8} e
BUSo 22	-	+	+	-	13.10×10^{-10} ghi
BUSo 23	-	+	+	+	5.90×10^{-8} hi
BUSo 24	-	+	+	-	5.16×10^{-10} i
LSD (0.05)	-	-	-	-	0.22

Means followed by uncommon letters are statistically different from each other at 5% level of provability by DMRT

*Nd= Not detected

Plate.1 Cellulase activity assayed after 24 hours incubation in Jensen-CMC plate the clear halo zone indicates cellulose degradation



Plate.2 Phosphate solubilizing activity assayed after 5 days incubation in Pikovskaya plate. The formation of clear zone around the microbial colonies indicated phosphate solubilization.

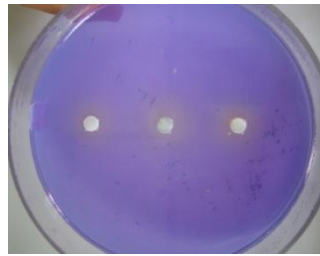
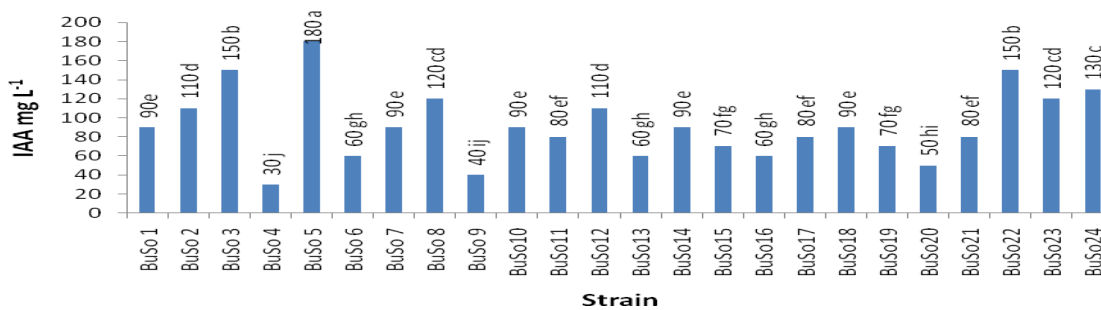


Plate.1 Appearance of red colour indicating IAA production by Diazotrophs



Figure.1 IAA production of the isolated diazotroph from sugarcane soil-plant system
Vertical bars represent standard error of treatment means



Means followed by uncommon letters are statistically different from each other at 5% level of provability by DMRT

Figure.2 Quantification of bacterial genomic DNA of *Acinetobacter calcoaceticus* (BU So 9) and *Bacillus cereus* (BU So 13) compare with 1 kb DNA ladder (AccuLadder, BIONEER) as a Marker (L1)

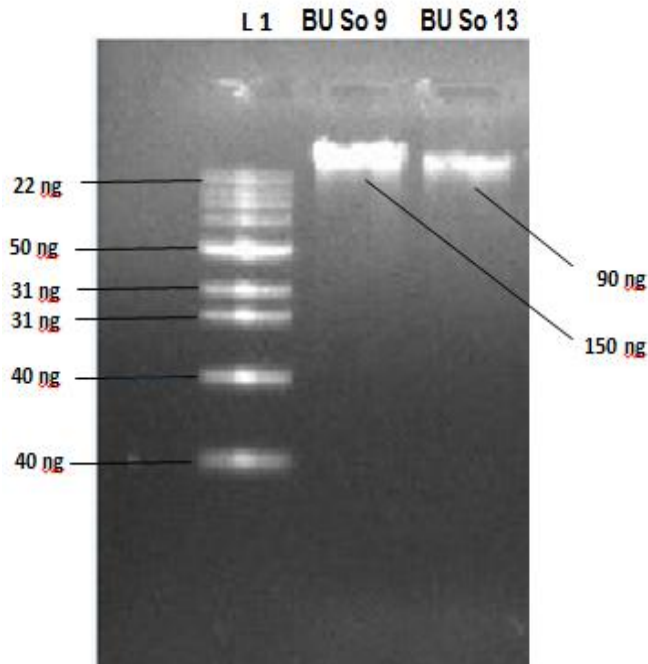


Figure.3 PCR amplification of *Acinetobacter sp.* Isolate (BU So 9) and *Bacillus sp.* Isolate (BU So 13)

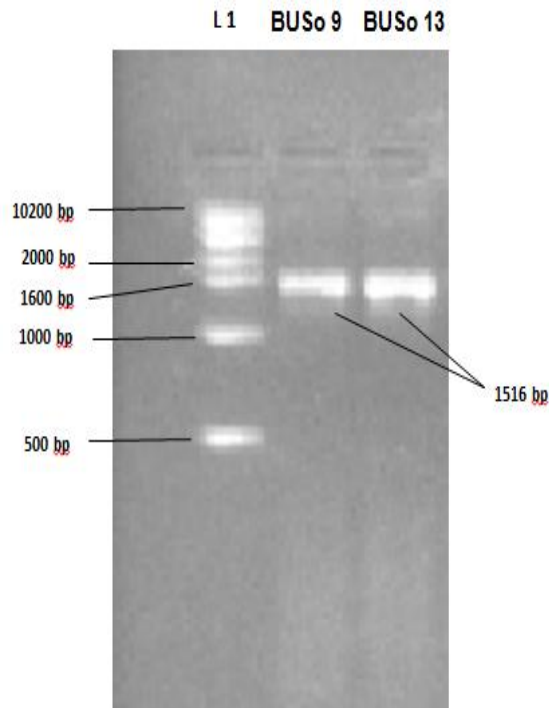


Figure.4 Phylogenetic tree of diazotrophic isolates BUSo 9

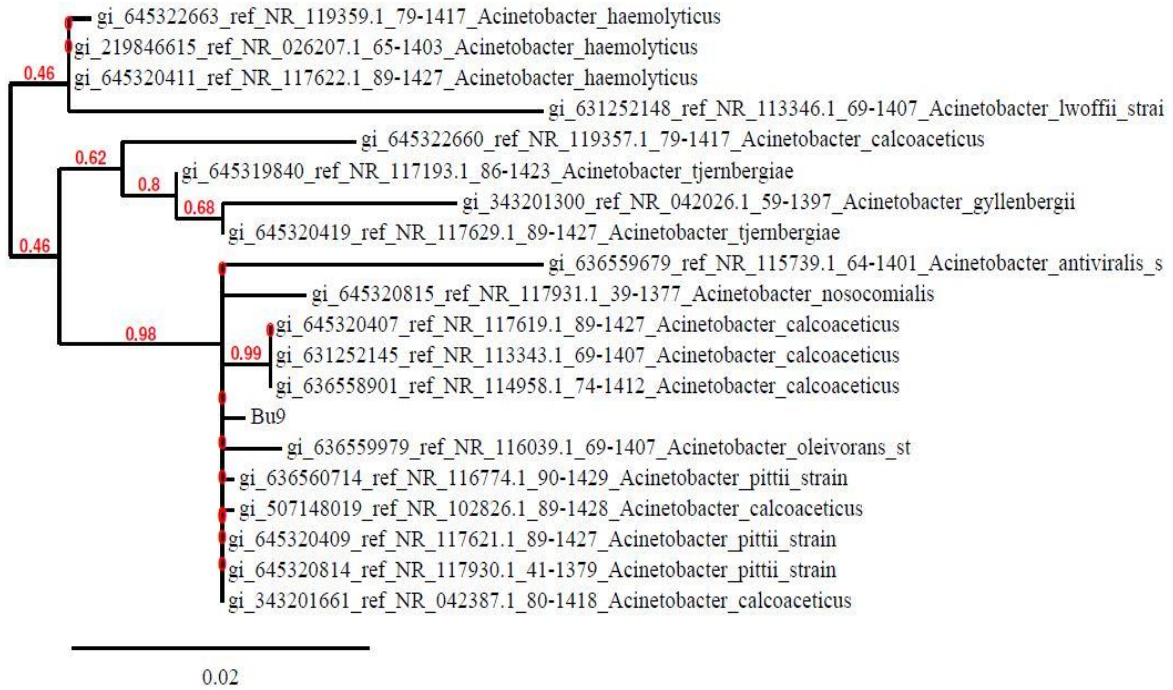
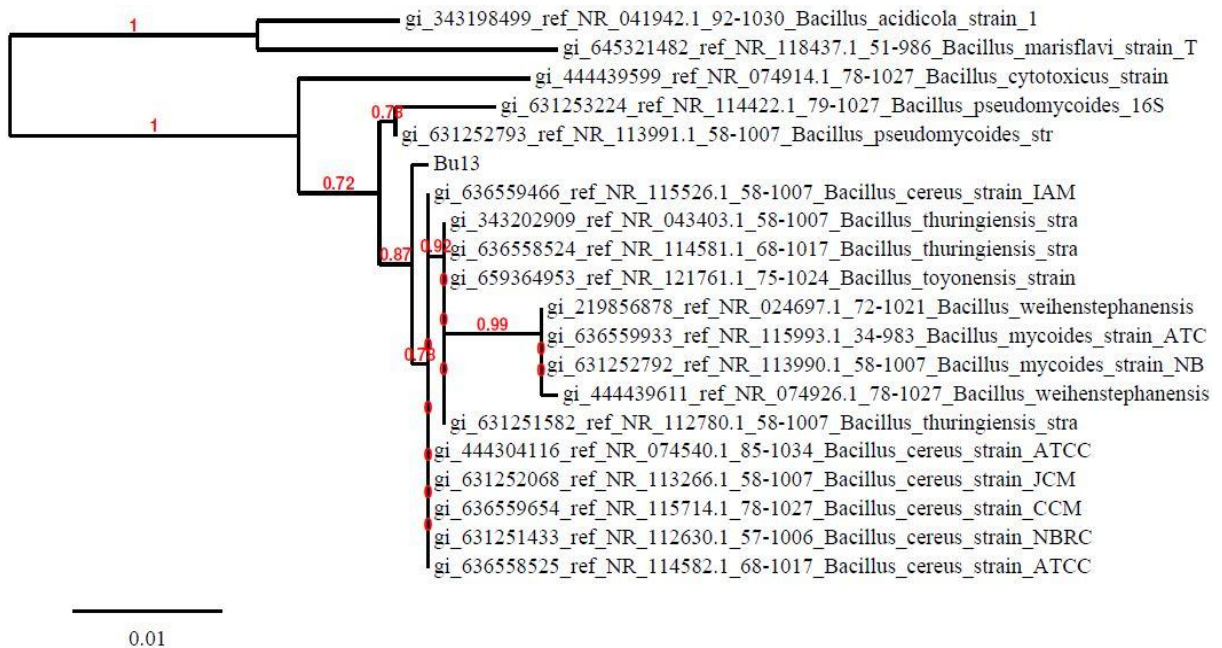


Figure.5 Phylogenetic tree of diazotrophic isolates BUSo 13



From 16S rDNA sequence analysis of the strains it was observed that 99 percent sequence identity was observed between the 16S rDNA sequence of BUSo 9 and

Acinetobacter calcoaceticus (Fig 4) and the 16S rDNA sequence of BUSo 13 and *Bacillus cereus* (Fig. 5).

Isolation of all the above bacteria was already reported from different crops by many researchers (Orhan *et al.*, 2006, Rodriguez & Fraga, 1999; Sturz and Nowak, 2000; Sahin *et al.*, 2004, Kang *et al.*, 2012). Some researcher reported that free-living N₂-fixing bacteria belonging to the genera *Beijerinckia*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Derrxia*, *Enterobacter*, and *Erwinia* appear to be frequent colonizers of sugarcane (Döbereiner and Ruschel 1958; Arias *et al.*, 1978; Hegazi *et al.*, 1979; Purchase, 1980; Rennie *et al.*, 1982; Graciolli *et al.*, 1983; Seldin *et al.*, 1984).

Based on this result, strains BUSo 9 and BUSo 13 were identified as *Acinetobacter calcoaceticus* and *Bacillus cereus*, respectively. Under *in vitro* conditions, both of the strains exhibited a high nitrogenase activity and produced a substantial amount of IAA. Chakraborty *et al.*, (2006) reported *Bacillus sp.* from tea rhizosphere is able to produce IAA and thus helps in the plant growth promotion. Husen, (2003) observed that bacterial strains *Bacillus cereus* when tested *in vitro* are found to solubilize phosphate and thus help in growth of plant. Çakmakçi *et al.*, (2006) observed that three different *Bacillus* isolates fixed nitrogen and increased growth in sugarbeet in a greenhouse study. Dadook *et al.*, (2013) reported *Acinetobacter calcoaceticus* as a nitrogen fixing diazotrophs which was isolated from asparagus rhizosphere. Previously research studies on *Acinetobacter* focused on growth potential, indole-3-acetic acid (IAA) production, inorganic phosphate solubilization, and nitrogen fixation (Huddedar *et al.*, 2002; Indiragandhi *et al.*, 2008; Khan *et al.*, 2011b).

From the above results it may be concluded that among twenty four bacterial strains 21 isolates were found as ARA positive. The highest ARA was found in strain

Acinetobacter calcoaceticus (BUSo 9) ($3.74 \times 10^{-4} \mu\text{mol C}_2\text{H}_4 \text{ cfu}^{-1}\text{hr}^{-1}$) which was statistically similar to *Bacillus cereus* (BUSo 13) ($1.54 \times 10^{-4} \mu\text{mol C}_2\text{H}_4 \text{ cfu}^{-1}\text{hr}^{-1}$). All of the strains were found as IAA producing. Among twenty-four, thirteen strains were found as Gram negative and eleven were Gram positive. Seventeen of the strains were positive for cellulose activity. Fifteen of the strains were found as positive for phosphate solubilization activity. For identification of bacteria from the isolated strains two promising strains were selected for 16S rDNA sequencing. From 16S rDNA sequence analysis of the strains it was observed that ninety nine percent sequence identity was observed between the 16S rDNA sequence of BUSo 9 and *Acinetobacter calcoaceticus* and also ninety nine percent sequence identity was observed between the 16S rDNA sequence of BUSo 13 and *Bacillus cereus*.

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