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CHARACTERIZATION OF DIAZOTROPHIC RHIZOBACTERIA UNDER VARIOUS CONDITIONS

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Abstract

Nitrogenase activity was analysed after supplementation of various carbon and nitrogen sources in the growth medium by Acetylene Reduction Assay methods in selected 9 isolates from 74 diazotrophic isolates. Enhancement in nitrogenase activity was recorded many fold by the addition of different organic carbon sources in which maltose and pyruvate showed better result than others. In case of supplementation of nitrogen sources, reduction of nitrogenase activity was observed. Nitrogenase activity increased from 22.7 to 72.7% in various strains when they are tested in anaerobic condition, Amplification of fragment of 390 bp showed that nitrogenase activity due to presence of *nifH* gene. Sequences were submitted to NCBI GeneBank and the accession number of *nifH* sequence of ECI-10A (FJ032023), AF-4B (FJ032024), AF-4C (FJ032022) and BN-2A (FJ032021) has been obtained. Phylogenetic analysis based on showed that these 4 isolates belong to the member of γ-proteobacteria, but show appreciable genetic diversity.

Key words: Diazotrophic bacteria, Rhizobacteria, Nitrogen fixation, Acetylene reduction assay, *nifH* gene, nitrogenise.

Introduction

Nitrogen is one of the most common nutrients required for plant growth and productivity as it forms an integral part of proteins, nucleic acids and other essential bio-molecules (Bockman, 1997). More than 78 % of N_2 is present in the atmosphere, but is unavailable to plants. It needs to be converted into ammonia, a form available to plants and other eukaryotes. Atmospheric N_2 is converted into forms utilized by plants by three different processes, (i) conversion of atmospheric nitrogen into oxides of nitrogen in the atmosphere (ii) industrial nitrogen fixation using catalysts and high temperature (300-500ºC) to convert nitrogen to ammonia, and (iii) biological nitrogen fixation involves the conversion of N_2 to ammonia by microorganisms using a complex enzyme system identified as nitrogenase (Kim and Rees, 1994). Biological nitrogen fixation contributes about 60% of the earth's available nitrogen and represents an economically beneficial and environmentally sound alternative to chemical fertilizers (Ladha *et al.*, 1997). The availability of nitrogen often limits plant growth in terrestrial ecosystem. It affects the productivity and the species composition of plant communities and ecosystem processes at all scales. In agriculture, nitrogen is one of the most widely used fertilizers, with a still increasing global input. The only biological reaction counterbalancing the loss of N from soils or ecosystems is biological nitrogen fixation.

Any bacterium can be considered as a rhizospheric diazotroph if: (i) it can be isolated from rhizosphere of the plant, and (ii) it does fix nitrogen, as demonstrated by acetylene reduction and/or ^{15}N-enrichment. Since the first report of N_2 fixation by *Clostridium* sp., the list of N_2-fixing microbes has increased tremendously (Ladha and Reddy, 2000). In general the well authenticated N_2-fixing organisms are all prokaryotes and can, for convenience, be divided into 3 main groups; (1) the free-living forms: these include heterotrophs, chemoautotrophs and phototrophs; (2) the symbiotic forms such as *Rhizobium* and *Frankia* where the presence of the host is usually essential for nitrogenase activity to occur; and (3) the associative symbiosis- those between cyanobacteria and eukaryotes, and between bacteria and tropical grasses where the symbiotic prokaryotes can also fix N_2 in the free-living state (Baldani *et al.,* 1997). Notably much information has been gathered from all three groups,

nevertheless, emphasis has always been focused on legume-*Rhizobia* symbiosis, the reason being agriculturally important crops. Several attempts have been made to transfer N_2-fixing ability from *Rhizobia* to other non-leguminous plants but little, if any, success has been attained so far (Ladha and Reddy, 2000). The present study focus on the diazotrophic bacteria isolated from rice rhizosphere of fields of Indo-Nepal border for the study of nitrogenase activity under various conditions including carbon and nitrogen sources and their genetic diversity based on *nifH* gene.

Materials and Methods

Bacterial strains and culture conditions

Diazotrophic rhizobacteria isolated from different rice fields of border area of India and Nepal were taken for this study. The method of isolation and culture conditions previously documented (Shrivastava UP and Kumar A, 2011; Shrivastava UP, 2013).

Acetylene reduction assay

Diazotrophic nature of various isolates was tested by the measurement of nitrogenase activity. Nitrogenase activity was estimated by acetylene reduction assay (Stewart *et al*., 1967). Overnight grown cultures (1.5 mL) in LB medium was spun and washed carefully with sterilized TDW to remove sources of combined nitrogen, if any, and then suspended in 100 µL of PBS (phosphate-buffered saline). 10 µL from this inoculum was added in 3 mL semi-solid (0.15 % agar w/v) $JNFb^-$ medium in a 7 mL vacutainer tube (Becton-Dickinson, Rutherford, NJ, USA) and grown for 4 days. Thereafter pure acetylene gas was injected in each tube by a hypodermic syringe to attain 10% final concentration. All the assays were performed at 30°C without shaking. The ethylene formed was analyzed in a 5700 Nucon Gas Chromatograph (Nucon Engineers Ltd., New Delhi) fitted with Porapak R column and flame ionization detector. N_2 was used as the carrier gas. Nitrogenase activity (acetylene reduction) was expressed in terms of µ moles C_2H_4 /mg protein /h.

Amplification of *nifH* gene

Amplification of *nifH* gene, one of the structural genes of *nif* operon was amplified by the method of Ueda *et al.* (1995a). Amplification was done in a PTC-100 Thermal Cycler (MJ Research, Inc., Walthon, MA, USA). Primers used for the amplification were 5'-GCI WTY TAY GGI AAR GGI GG-3' for 19 F and 5'-AAI CCR CCR CAI ACI ACR TC-3' for 407 R (where I = inosine, R = A or G, W = A or T, and Y = C or T). Genomic DNA was extracted using DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) as per the instructions of manufacturer. PCR reaction mix contained 1X *Taq* DNA polymerase buffer, 20 pmole of each primer, 25 mM each dNTPs, 1 U *Taq* DNA polymerase and 50 ng of template DNA in a final volume of 25 µL. DNA of *E. coli* was taken as a negative control. Thermal cycle was set as; initial denaturation at 94°C for 3 min, 94°C for 30 s, 56°C for 1 min and 72°C for 30 s (steps 2 to 4 for 35 cycles) followed by extension at 72°C for 5 min and storage at 4°C. The amplified product was electrophoresed in a 1.5 % agarose gel and monitored under UV light in a Gel Documentation unit (Bio-Rad Laboratories, USA). PCR amplified product of *nifH* (390 bp) was purified to remove unused primers and dNTPs with QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) as per the instructions of manufacturer.

Results and Discussions

Response of nitrogenase activity towards organic carbon sources

The growth of all the isolates is influenced by the addition of different organic carbon sources in the medium; it was desirable to assess their impact on nitrogenase activity. It is evident from the data of Fig. 1 that nitrogenase activity is indeed affected by the addition of different organic carbon sources. However the response was differential in different isolates with different carbon sources. Among all the carbon sources, the highest enhancement in activity was observed with the addition of maltose and pyruvate (4-7 folds) followed by sucrose and fructose (1.9 to 6.6 folds). Other carbon sources namely succinate, formate and acetate showed 1.5 to 5 folds increase in various isolates. Interestingly, there was absence of stimulation in the presence of glucose in almost all the isolates (Fig. 1).

Nitrogenase activity under anaerobic condition

In general nitrogenase activity of aerobic N_2-fixing bacteria shows multi fold stimulation if grown or preincubated under anaerobic condition. Keeping this fact in mind, we tested response of nitrogenase activity of all the isolates following preincubation under 95% argon (Ar) + 5% CO_2 for 24 h. Nitrogenase activity was significantly stimulated in all the isolates. It is evident from the data of Fig. 2 that the highest increase in activity was observed in AF-4B (72.7%) and the least increase was by ECI-12A (22.7%).

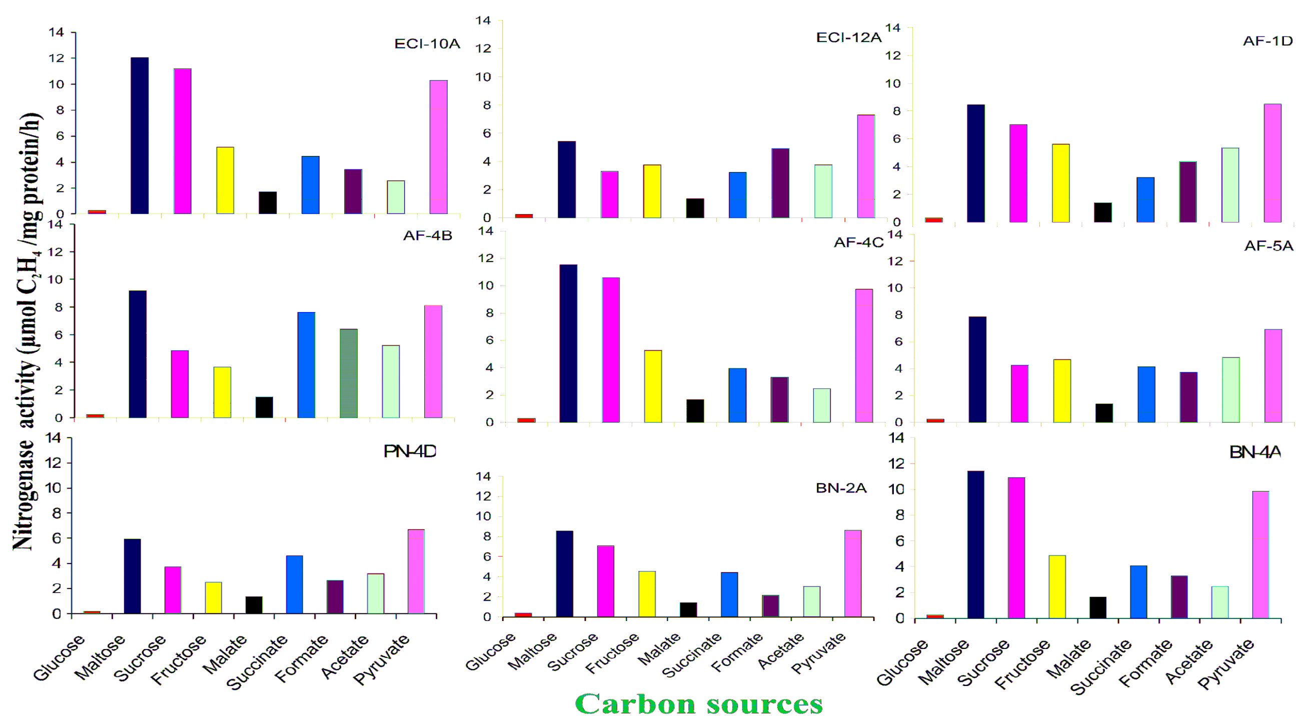

Fig. 1: Response of nitrogenase activity towards various organic carbon sources. Nitrogenase activity was measured after 96 h growth. Cultures were grown in JNFb⁻ medium supplemented with various organic carbon sources. Final concentration of different carbon sources used was: glucose, fructose, sucrose and maltose-1%; succinate, pyruvate and malate- 0.5% and acetate-0.1%. In each tube 10% acetylene (final concentration) was injected and ethylene formed was measured after 4h incubation at 30°C. Results are based on average of three replicates.

Table 1: Effect of various amino acids on nitrogenase activity

Bacterial isolates	Nitrogenase activity (µmol C_2H_4/mg protein/h)						
	Amino acids						
	Control	Ala	Gln	Asp	Arg	His	Glu
ECI-10A	1.72	1.21	0.58	1.63	0.81	0.97	0.73
	(0)	(29.6)	(66.2)	(5.2)	(52.9)	(43.6)	(57.5)
ECI-12A	1.35	0.84	0.35	0.98	0.66	0.83	0.31
	(0)	(37.8)	(74.1)	(27.4)	(51.1)	(38.5)	(77.0)
AF-1D	1.41	0.93	0.37	1.21	0.69	0.87	0.32
	(0)	(34.0)	(37.8)	(14.2)	(51.1)	(38.3)	(77.3)
AF-4B	1.53	1.05	0.45	1.35	0.82	0.98	0.39
	(0)	(31.4)	(70.6)	(11.8)	(46.4)	(35.9)	(74.5)
AF-4C	1.65	1.02	0.46	1.31	0.76	1.12	0.64
	(0)	(38.2)	(72.1)	(20.6)	(53.9)	(32.1)	(61.2)
AF-5A	1.38	0.91	0.33	1.15	0.67	0.88	0.29
	(0)	(34.1)	(76.1)	(16.7)	(51.4)	(36.2)	(79.0)
PN-4D	1.33	0.85	0.28	1.02	0.64	0.80	0.24
	(0)	(36.1)	(78.9)	(23.3)	(51.9)	(39.8)	(82.0)
BN-2A	1.42	0.95	0.39	1.26	0.72	0.89	0.35
	(0)	(33.2)	(72.5)	(11.2)	(49.3)	(37.3)	(75.3)
BN-4A	1.63	1.13	0.59	1.36	0.91	0.99	0.56
	(0)	(30.6)	(63.8)	(16.5)	(44.2)	(39.2)	(65.6)

Actively growing cultures were used for the estimation of nitrogenase activity. Cultures were grown in JNFb⁻ medium supplemented with desired amino acid.
Final concentration of each amino acid was 0.5 mM.
Value shown in parentheses indicates percent inhibition of nitrogenase activity.
Results are based on average of three experiments conducted separately.

Effect of inorganic combined nitrogen sources

It is evident from the data of Fig. 3 that there is a gradual loss of nitrogenase activity with increasing concentrations of NH_4Cl in all the isolates. Interestingly, some enhancement of nitrogenase activity in the presence of 0.1 mM of NH_4Cl and 0.1 mM of $Ca(NO_3)_2$ was observed in AF-4C in the initial phase however there was decrease with time of incubation. Altogether, there was inhibition of activity with as low as 0.1 mM NH_4Cl, the complete inhibition was attained with 0.5 mM in all the isolates. Similar to the effects of NH_4Cl, $Ca(NO_3)_2$ also caused inhibition of activity but at a higher concentration. Contrary to complete inhibition caused by NH_4Cl at 0.5 mM, $Ca(NO_3)_2$ could not abolish nitrogenase activity at this concentration in any isolates (Fig. 2).

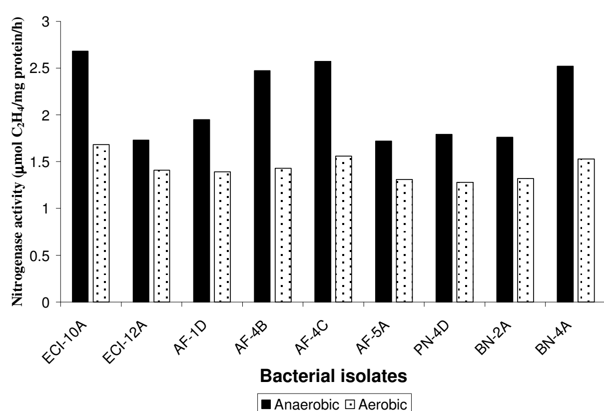

Fig. 2: Nitrogenase activity of various isolates under anaerobic (95% Ar + 5% CO_2) and aerobic condition. Cultures were preincubated in 95% Ar + 5% CO_2 for 24h and then C_2H_2 reduction was estimated under 90% Ar + 10% C_2H_2. Control (aerobic) cultures were grown in semi-solid medium as described in materials and methods section. Activity was measured after incubation under identical conditions.

Response of nitrogenase activity towards amino acids

Once it became apparent that inorganic combined nitrogen sources show inhibitory effect on nitrogenase activity, it was desirable to test the effects of various amino acids on the activity. Among all the amino acids tested, maximum inhibition was observed by the addition of glutamic acid and glutamine (ranging from 37.8 to 82.0%) and the lowest inhibition (ranging from 5.2 to 27.4%) was noted by aspartic acid in different isolates. Other amino acids such as alanine, arginine and histidine also showed significant inhibition (29.6 to 53.9%) (Table 1).

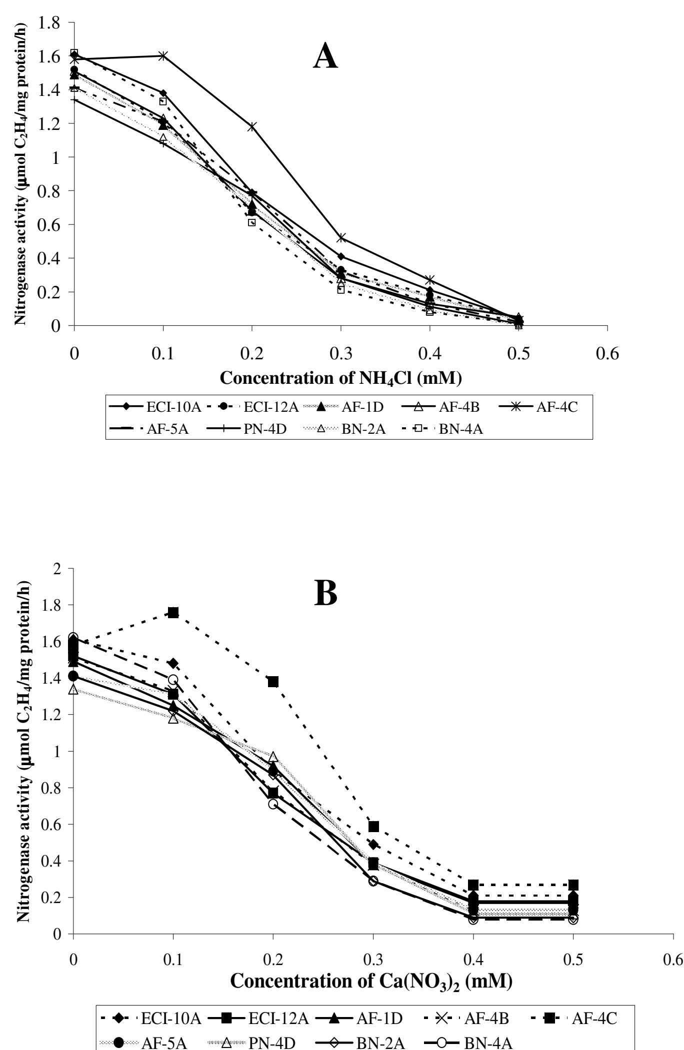

Fig. 3: Effect of NH_4Cl **(A)** and $Ca(NO_3)_2$ **(B)** on nitrogenase activity. Cultures were grown in $JNFb^-$ medium supplemented with desired concentrations of NH_4Cl or $Ca(NO_3)_2$ for 12 h and thereafter incubated with C_2H_2 (10%) for 6 h and then C_2H_2 reduction assay was made. Results are based on average of three experiments conducted separately under identical conditions.

PCR amplification of *nifH* gene

Results obtained above clearly showed that all the putative diazotrophic isolates have active nitrogenase activity and thereby seem to be potential N_2 fixer, it was desirable to confirm this character employing molecular approach. Accordingly, an attempt was made to amplify *nifH* (390 bp) gene which encodes dinitrogenase reductase (component II) of all the nitrogen-fixing bacteria. Results showed amplification of ~390 bp fragment of *nifH* gene in all the isolates.

Sequencing of *nifH* gene & phylogenetic analysis

nifH gene of four potential isolates (ECI-10A, AF-4B, AF-4C and BN-4A) which showed higher nitrogenase activity (more than 1.5 µMol C_2H_4 formed/mg protein/ h) was sequenced after purification of PCR product in Applied Biosystem 310 Genetic Analyzer adopting standard sequencing protocol. Sequences were submitted to NCBI GeneBank and the accession number of *nifH* sequence of ECI-10A (FJ032023), AF-4B (FJ032024), AF-4C (FJ032022) and BN-2A (FJ032021) has been obtained. *nifH* gene sequences of the above four isolates were analysed for the construction of phylogenetic tree together with *nifH* gene sequence of 56 known diazotrophic strains of bacteria and 5 *nifH* gene fragments of environmental samples retrieved by NCBI GenBank. Phylogenetic analysis showed that AF-4C (FJ032022) and BN-4A (FJ032021) were very closely related, whereas ECI-10A (FJ032023) shared homology with AF-4B (FJ032024). Analysis of *nifH* sequences suggests that these 4 isolates belong to the member of γ-proteobacteria, but show appreciable genetic diversity (Fig. 5).

Diazotrophic bacteria have been isolated from rhizosphere in the early sixties but their biological nitrogen fixation (BNF) contribution to the sustainable agriculture system is stil1 under debate (Dobbelaere *et al.*, 2003). Much evidence has pointed towards the role of phytohormones, because they are able to change the root morphology and growth of the plant and thus considered as PGPR (Bashan *et al.,* 2004). Nevertheless, there is strong evidence of nitrogen accumulation in the plants due to activities of rhizosphere nitrogen-fixing bacterium based on ^{15}N isotope dilution technique (Boddey *et al.,* 1983). It has been demonstrated that about 20 kg $ha^{-1}y^{-1}$ of the N accumulated in *Paspalum notatum cv. batatais* was derived from BNF.

In this study initial test made by acetylene reduction assay revealed detectable level of nitrogenase activity in all the 74 isolates pointing towards their diazotrophic nature. This presumption is based on the fact that acetylene reduction assay is a standard and routine technique for the estimation of nitrogenase activity in all the nitrogen-fixing organisms including cyanobacteria and bacteria (Stewart, 1980; Ladha and Reddy, 2000; Eckert *et al.,* 2001). However, other methods such as N balance and ^{15}N methods are considered more appropriate and sensitive methods (Ladha and Reddy, 2000). Nine isolates namely ECI-10A, ECI-12A, AF-1D, AF-4B, AF-4C, AF-5A, PN-4D, BN-2A and BN-4A (selected solely on the basis of efficient growth promoting activities) were chosen for testing essential features of nitrogenase activity in response to physical and nutritional factors so as to make comparison with other nitrogen-fixing bacteria vis-à-vis confirmation of diazotrophy. It is known that a variety of carbon sources accumulate in rice roots during active growth, which might be used by rhizospheric bacteria for growth and other vital metabolic activity (De Los Santos *et al.*, 2001). Obviously rhizospheric bacteria must respond to these carbon sources upon growth *in vitro* condition. Results of this study showed that addition of carbon sources viz., maltose, sucrose, fructose and pyruvate to the medium not only supported better growth rather nitrogenase activity was also significantly stimulated in almost all the isolates. Stimulation of nitrogenase activity seems to be due to enhanced growth as well as availability of ready-made carbon source in the medium for the high energy consuming reaction of N_2 fixation. Lack of stimulation by certain carbon sources might be due to lack of uptake or absence of certain enzymes responsible for the metabolism under nitrogen-fixing condition. It is evident from the data that glucose either showed very poor nitrogenase activity in majority of the isolates. Inability to reduce acetylene when glucose was the carbon source has also been observed by De Los Santos *et al.*, (2001). Presence of high level of nitrogenase activity in all the isolates with pyruvate might be due to preference for sugar acid since it is always present in the plant's roots and their surroundings (Ladha and Reddy, 2000).

Acetylene reduction assay (ARA) is an undoubtedly an indirect but excellent method to test the diazotrophic nature of any microorganism, but it is felt that cultures showing high acetylene reduction activity may not be always associated with N_2-fixing ability and the rate of ARA may not be always correlated with actual rate of N_2 fixation (Malik *et al.,* 1991; Stoltzfus and de Bruijn, 2000). It has been also reported that nitrogen-fixing cultures of bacteria or cyanobacteria show very high level of ARA under argon + CO_2 atmosphere even in the absence of traces of N_2. In addition to tests made by diazotrophic growth and ARA, further confirmation was made by the amplification of 390 bp segment of *nifH*, which is one of the three structural genes (*nifHDK)* of nitrogenase polypeptide (Ueda *et al.*, 1995a). Our results showed that all the putative diazotrophic isolates do have 390 bp fragment of *nifH* which was evident in standard PCR assay. To rule out the possibility of erroneous amplification, a positive and a negative control were always used. Previous studies employing different *nifH* PCR primers also have shown successful and specific amplification of *nifH* segments from a variety of organisms and natural samples (Rosado *et al.*, 1998; Widmer *et al.,* 1999).

Amplification of *nifH*, *nifD*, and *nifK* by PCR or RT-PCR has been frequently employed in detection of nitrogen-fixing ability of the bacterial and cyanobacterial isolates taken either from laboratory grown culture or directly from environmental samples (Ueda *et al*., 1995a, b; Zehr *et al*., 1998; Auman *et al.,* 2001; Egener *et al.,* 2001; Minerdi *et al.,* 2001). Identity and homology of *nifH* gene in the isolates reported in this study are further confirmed by sequence analysis. Fidelity of the amplified segment of *nifH* was evident from the sequence, which displayed similarity with *nifH* sequence available in database of GenBank. Physiological and molecular evidences gathered with all the isolates clearly suggest that these isolates are indeed diazotrophic and are capable of N_2 fixation.

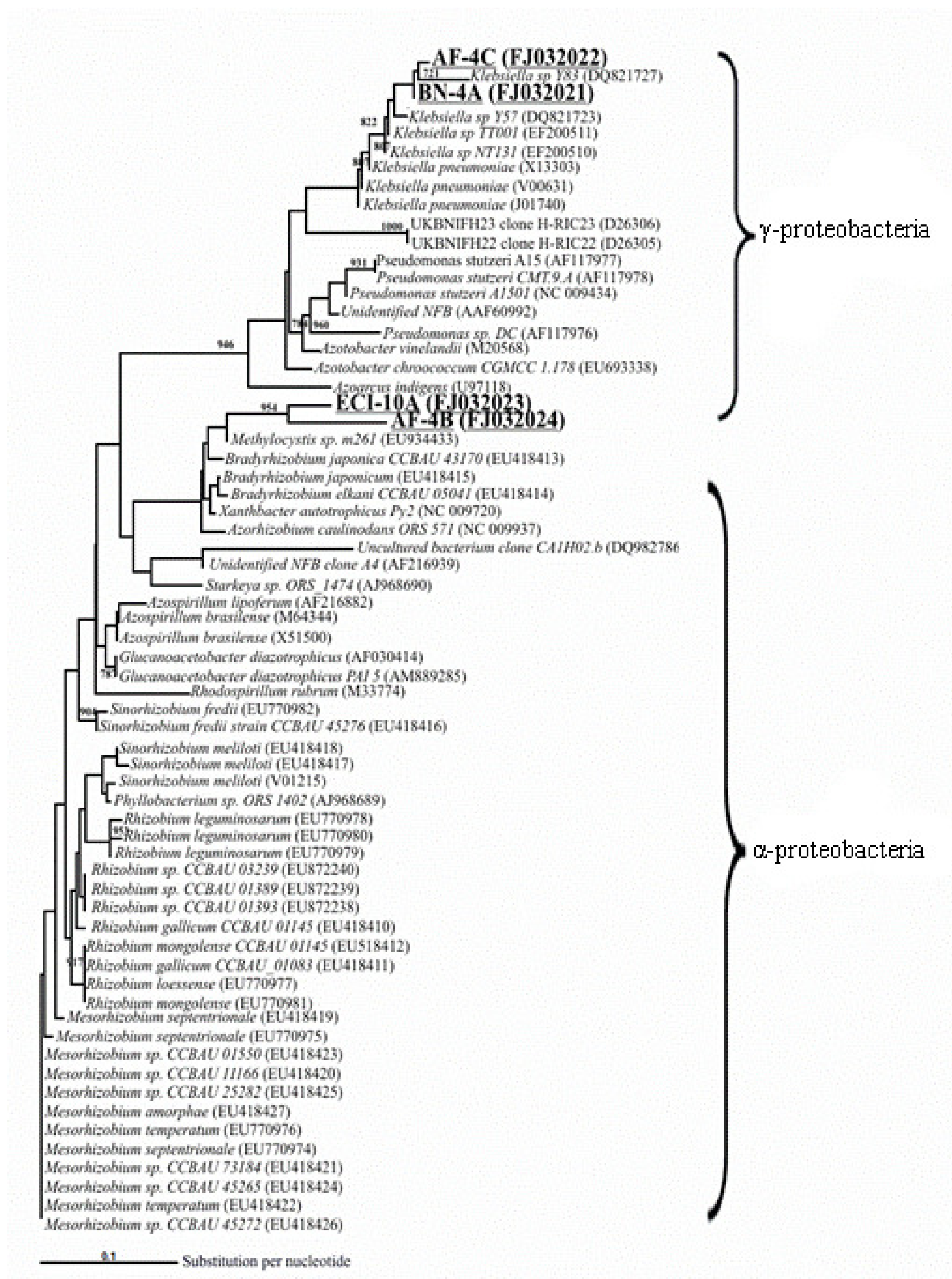

Fig. 3: Phylogenetic tree of *nifH* gene sequence based on deduced 130 amino acids sequence using neighbour-joining method. Bootstrap values over 70% in 1000 interactions are shown at the node. The GeneBank accession number of each sequence is also shown.

Additionally four potential isolates showing higher nitrogenase activity were further characterized by *nifH* sequence based on phylogenetic analysis along with 56 known sequences from the database of GeneBank. Phylogenetic analysis showed that these 4 isolates belong to the member of γ-proteobacteria; where AF-4C (FJ032022) and BN-4A (FJ032021) were closely related, and ECI-10A (FJ032023) showed homology with AF-4B (FJ032024). Analysis of *nifH* sequence suggested appreciable genetic diversity among these four isolates, which is in agreement with the previous report (Ueda *et al*., 1995b). It would be desirable to amplify and sequence *nifH* fragment of a large number of PGPR so as to have better understanding of molecular diversity at the level of *nifH* gene.

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