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Molecular Characterization of Free Living N_2 Fixing Bacteria Isolated from Agricultural Soils of North Gujarat, India

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ABSTRACT

Background: Molecular identification of a wide range of organisms capable of carrying out biological nitrogen fixation (BNF) are diverse in nature and significantly improves plant growth. Biological N_2 fixation reflects the activity of a phylogenetically diverse list of microorganisms. Molecular characterization provides efficient means to identify organisms with the potential of N_2 fixation. Applying these techniques in an array of environments has considerably broadened our understanding of the suite of organisms that can carry out BNF.

Methods: Thirty-four strains of free living N_2 fixing bacterial strains were isolated from diverse plants cultivated in North Gujarat, including wheat, cotton, castor and pearl millet, using a nitrogen-free selective medium. Acetylene reduction assay was used to check the ability of all bacteria to fix nitrogen. Hybridization with *nif*H probe derived from *Azotobacter vinelandii* with isolated free-living nitrogen-fixing bacteria showed a positive result. The selected strains were characterized by molecular analysis like; ARDRA and 16S rDNA sequencing.

Result: Based on molecular characterization 17 strains to known groups of nitrogen-fixing bacteria, including organisms from the genus *Azotobacter*, *Pseudomonas, Enterobacter, Arthrobacter, Bacillus, Variovorax, Nocardiodies, Rhodococcus, Mycobacterium, Planococcus, Microbacterium* have been identified. One of the strains was identified as unknown bacteria. The potential strains were identified by 16srDNA analysis and also corroborated by morphological and biochemical characterization.

Key words: 16S rDNA analysis, ARDRA, Free living diazotrophs, Nitrogen free medium.

INTRODUCTION

Nitrogen is the universal restricting component for the development and productivity of plants in terrestrial ecosystems (Stein *et al.* 2016). Although dinitrogen is the most plentiful component in the air, it is biochemically inaccessible for plants and most microorganisms as they utilize only reduced or oxidized forms of nitrogen (Kennedy and Islam 2001). The two atoms in dinitrogen are triple-bonded, a significant energy level of energy to dissociate and reduce to ammonia (Figg *et al.* 2012). A few natural systems can convert dinitrogen into other utilizable forms of reactive nitrogen, primarily, nitrite and nitrate and to integrate ammonia into organic compounds, mainly, amino acids (Chianu *et al.* 2011). The atmospheric nitrogen can be fixed by two natural processes: Lightning and biological nitrogen fixation. Lightning makes about 1% ammonia of the net nitrogen fixed per year (Santi *et al* 2013). The group of microorganisms distributed among bacterial and archaeal domains can fix nitrogen (Vitousek *et al.* 2002) and fixes about half of the total nitrogen per year (Igarashi and Seefeldt 2003). Biological nitrogen fixation is both cost-effectively and ecologically helpful for sustainable agricultural production (Peoples and Craswell 1992). Nitrogen fixation is carried out under different conditions: Separately, in free association with other organisms or in stringent symbiosis with them, such as in the *Rhizobium*-legume-plant symbiosis. The symbiotic nitrogen fixation is the most capable kind of relationship between diazotrophic microorganisms and plants and is significant for some farming practices (Van Heerwaarden, 2018).

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Microorganisms can build up either mutualistic or pathogenic relationships with the plant. The molecular mechanisms for the communication between micro organisms and plant are very much similar although the outcome is entirely different. In particular, symbiotic nitrogen-fixing bacteria of legume plants, collectively known as rhizobia and phytopathogenic bacteria have adopted similar strategies and genetic traits to colonize, invade and establish a chronic infection in the plant host (Fox, *et al.*, 2006). Despite symbiotic microbes serving as an influential group for nitrogen fixation, free living nitrogen fixers additionally contribute a considerable measure of nitrogen to different environments (Kahindi *et al.* 1997; Unkovich and Baldock

2008). Free living nitrogen fixers include various heterotrophs including members of Proteobacteria, Firmicutes, Archaea and Cyanobacteria (Stewart *et al.* 1969). They utilize the energy from the oxidation of organic molecules. Free-living diazotrophs have been considered to contribute to a low measure of total fixed nitrogen in contrast with the symbiotic nitrogen fixation (Stacey 1997). *Acetobacter*, *Azotobacter*, *Campylobacter* and *Pseudomonas* have also been reported for free living nitrogen fixation (Weber *et al*, 1999). Molecular identification by using *nif*H gene analysis can be accomplished for many unknown nitrogen-fixing organisms. It is also helpful to analyze their genetic potential for the nitrogen fixation (Wei *et al.,* 2008). The n*if*H genes can be employed as markers for detecting and studying the genetic diversity of diazotrophic organisms in microbial communities, like those in rice roots (Ueda *et al.*, 1995) or forest soil (Widmer *et al.*, 1999). Putative nitrogenase amino acid sequences revealed that more than half of the *nif*H products were derived from methylotrophic bacteria, such as *Methylocella* spp.

In this study, we have isolated several free-living nitrogen fixers from various agricultural plants and characterized them using phenotypic and genotypic methods to assess their taxonomic diversity and investigate their ability to fix atmospheric nitrogen and the occurrence of nifH-like genes.

MATERIALS AND METHODS

Isolation, morphological and biochemical characterization of free-living nitrogen-fixing bacteria

The soil samples were collected from agricultural plants in major district of North Gujarat; Mehsana, Patan, Sabarkantha and Banaskantha, Gujarat, India. All the experiments were conducted in the Research laboratory, Biotechnology Department, Mehsana Urban Institute of Sciences, Ganpat University from 2018-2019. One gram of rhizospheric soil sample was inoculated into a selective nitrogen free media Ashby's mannitol medium (Baldani *et al* 2014) and incubated at 30°C for 10 days. All the diazotrophic strains were isolated in pure form and maintained at 4°C for further study. The nitrogen fixation by the acetylene reduction test were performed after 24 hours of incubation in the presence of acetylene (Turner *et al* 1980). Strains were characterized by Gram's staining, colony morphology and motility. Biochemical tests were performed by using strips (HiMedia Pvt. Ltd.) and additional phenotypical tests were performed as described by Holt *et al.*, (1994), Hartmann and Baldani (2006) and Nemergut, *et al.* (2013).

DNA extraction

Chromosomal DNA was extracted using the genomic DNA extraction kit (Chromus Biotech Pvt. Ltd). The isolates were freshly grown on N_2 free agar media and were re-suspended into 10 ml of N_2 free broth, before being spun down and re suspended in 1 ml of N_2 free broth. Genomic DNA was isolated according to the protocol provided with the kit. DNA purification was also carried out (Page *et al* 1979).

Amplified ribosomal DNA restriction analysis (ARDRA)

The restriction enzymes to be used for ARDRA were selected by using virtual restriction of the 16S r-RNA gene sequences of various nitrogen fixing bacteria retrieved from the GenBank using the serial cloner software (Version 2.6). The 16S rDNA gene was amplified by means of universal bacterial primers for 35 reactions cycles. 1.5kb of PCR product was digested with restriction enzymes; *HpaII, TaqI* and *RsaI*. 05 units of each enzyme were mixed with 12.5 µl of the amplification product. The final product of restriction was incubated for 5 h at 37°C. The digests were analyzed by electrophoresis on a 2% agarose along with100 bp and 250 bp DNA ladder at 110V for about 2h 30 min and visualized to check the ARDRA pattern. ARDRA pattern was compared with the ARDRA profile of known reference strain of bacteria. The amplified DNA profiles were compared based on the presence (1) or absence (0) of fragments. (Reinhardt *et al* 2008).

16S rDNA sequencing and phylogenetic analysis

After amplification of DNA, the PCR product was sent for sequencing to chromus biotech pvt. Ltd., India. The ABI 3500 XL genetic analyzer was used for sequencing of the PCR product. The sequencing reaction was carried out by big dye terminator version 3.1" cycle sequencing kit. POP_7 polymer was used for 50 cm capillary array. The data analysis was carried out by seq scape_ v 5.2 software and the reaction plates used in the sequencing was applied Biosystem Micro Amp Optical 96 Well Reaction plates. The basic BLAST search was used for comparison of sequences (Altschul *et al.*, 1997) and then aligned using the ClustalW program (Larkin *et al.* 2007) available at the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw/) with the sequences retrieved from the GenBank database (Benson *et al.*, 2003) available at the NCBI website (http://www.ncbi.nlm.nih. gov/). The phylogenetic tree of all the isolates were generated. The similarity matrix was generated with Jaccard coefficient and the distance matrix was used for constructing the dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmetic Averages). The dendrogram was generated in Newick format.

Detection of *nif*H genes by Dot-blot analysis

Dot-blot hybridization assays were carried out using standard protocols described elsewhere (Hybond N+, ECLSystem, Amersham Pharmacia Biotech). A fragment of 705 bp corresponding to the initial 5′-end of *Azotobacter vinelandii nif*H gene was amplified by PCR using specific primers PPf (5′ GCAAGTCCACCACCTCC 3′) and PPr (5′ TCGCGTGGACCTTGTTG 3′). PCR conditions comprised: 30 ng of DNA in 25 µL reactions containing 1,5 mM MgCl2, 200 µM each deoxynucleoside triphosphates, 0.5 µM each primer and 1,5 U *Taq* polymerase (HiMedia Pvt. Ltd.) in 1 X *Taq* buffer. Amplification was performed with the following cycling program: Initial denaturation at 94°C for 3 min followed by 30 rounds of 94ºC for 30 s, 58°C for 30 s and 72°C for 45 s. A final extension of 72°C for 7 min. was used.

The *nif*H gene fragments were labelled by using the horseradish peroxidase kit (ECL system, amersham pharmacia biotech) and used as a probe in dot blot hybridizations. DNA preparations from strains Vis 2, Pr 2, Ha 2 and Hmt 4 were spotted onto Hybond N+ membranes and these were hybridized and detected according to the manufacturer's instructions (ECL system, amersham pharmacia biotech). High stringency hybridization conditions were used in the assays. Hybridization signals were detected by exposure to X OmatX-ray films.

RESULTS AND DISCUSSION

Morphological and biochemical characterization of free-living nitrogen-fixing bacteria

Microscopic observations of all the stained cells showed different nature and majority of strains were appearance of Gram negative, motile and short rod-shaped cells and some few strains were appearance as gram positive, coccoid shaped and filamentous type. On Ashby's Mannitol agar medium all the isolates produced different type of colonies; Isolates Am3 had Gummy, large, circular colonies; Isolate Bch3 showed small, circular, flat, white colonies; Isolate ch4 showed flat, rough, filamentous colonies; isolate Hmt4 showed small, translucent, circular, raised colonies; Isolate Rd1 showed Gummy, large, circular colonies; Isolate Vij2 showed Large, gummy, translucent and circular colonies. Isolates Am5 showed small, round, colonies and formed red pigment after 3-4 days. Isolate Idr2 showed Large, circular, due drop, convex colonies; Isolate Msn4 showed Gummy, large, circular colonies and formed Brownish pigment after 6-7 days; isolate Pln4 showed Small, round, colonies and formed red pigment after 3-4 days; Isolate Ptn4 showed small, circular colonies and formed yellowish pigment after 3-4 days; Isolate Trd 3 showed, flat, irregular colonies and formed Yellowish white pigment after 4-5 days; Isolate Vis2 showed gummy, wavy ,big colonies and formed Brownish pigment after 7-8 days; isolate Idr 4 showed small, round ,orange pigmented colonies; isolate kd 2 showed Gummy, medium and circular colonies; isolate Dt 3 showed medium, smooth, circular and white creamy colonies.

Biochemical characterizations of all the isolates were found to give positive reaction for oxidase test. Reduction of nitrate was shown by isolates Rd 1, Am3, Vij 2, Ch 4, Trd3, Msn4, Vis 2 and Pln4. Isolates Am 5, Idr2, Ptn 4, Vis 2, Hmt 4, Pln4, Vij 2 and Ch 4 produce ammonia. Isolates Msn4, Ptn 4 Vis 2 Hmt 4, Rd 1, Trd3 hydrolyzed urea. Isolates Msn4, Bch3, Vis 2, Hmt 4, Rd 1, Ch 4, Trd3 and Idr4 hydrolyze starch. Isolates Msn4, Vis2, Hmt4 and Vij2 produce IAA and isolates Msn4, Vis2, Hmt4, Vij2, Trd3 and Dt3 produce PHB as a cell reserve material. All the isolates were determined for their ability to grow in presence of 1% to 10% NaCl and between pH extremes of 3.5 and 12. isolates Am5 and Idr2 tolerate to the high pH (pH 11 and 12) while isolates Am 5, Msn4, Idr2, Bch3, Rd 1, Am-3 and Vij 2 tolerate low pH (pH 4.5). In the studies of salt tolerance characteristics, all the isolates are able to grow in 1% NaCl whereas one isolate Idr4 was able to grow in 9% NaCl. Isolates Ch4 and Trd3 were able to grow at 52°C while isolates Am5 and Idr4 were able to grow at 8°C while others were growing well at temperature between 31°C and 43°C. Carbohydrate utilization test showed that all the isolates utilized various carbohydrates like Sucrose and Mannitol. No isolates were found to show equal propensity to utilize all the carbohydrates. Within the isolates there were great variations. Only four isolates-Ch4, Idr4, Idr2 and Bch3 utilized lactose.

Isolation of nitrogen-fixing bacteria and detection of *nif*H related gene sequences

All the Free-living bacterial strains were isolated by using the selective media NFb (Hartmann and Baldani 2006). These strains reduced acetylene on the Gas chromatographic analysis performed, hence indicating their nitrogen fixation capability after the 24-hours period. The dot blot hybridization with a *nif*H probe was used to obtain further indications of their potential to fix nitrogen.

Dot blot hybridization (Fig 1) revealed the presence of *nif*H related sequences in DNA from strains Vis 2, Pr 2, Ha2 and Hmt 4 under the high stringency hybridization conditions used in the assays. The negative controls used, namely *E. coli* and human DNA, did not show any hybridization signal with the *nif*H probe, demonstrating the specificity of the used probe. Positive results in dot blot hybridization for strains Vis 2, Pr 2, Ha2 and Hmt 4 corroborate their ability for nitrogen fixation, suggested by the acetylene reduction assay.

ARDRA

Stringent PCR conditions allowed amplification of a single 16S r-DNA fragment. All isolates yielded a band of ~1.5 kb in size after amplification with the universal eubacterial primers. ARDRA with *TaqI*, *HpaII* and *RsaI* (4 base cutters) digestion resulted in the number of pattern type. The isolates

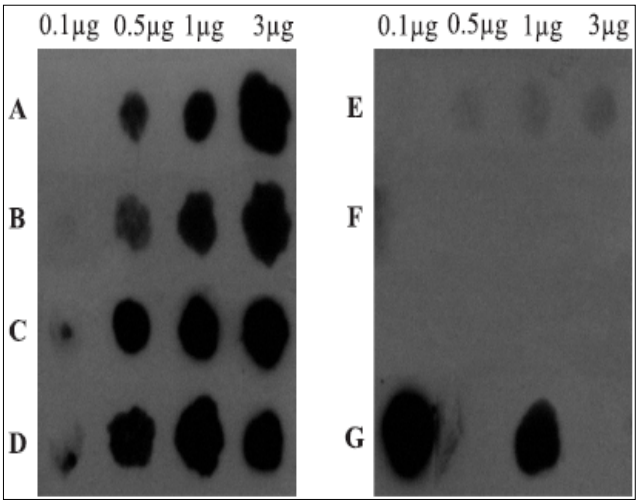

Fig 1: Nif gene detection by using *nif*H probe A) Genomic DNA from Vis2; B) Pr2; C) Ha2; D) Hmt4; E and F) Negative control; G) *A. vinelandii*(0.1µg) and *A. chrococcum* (1 µg).

were grouped into 17 ARDRA types (Table 1). From 17 ARDRA group, organisms belonging to 08 groups were identified using similarity with reference strains. ARDRA groups which were not identified by ARDRA were identified by 16S rRNA gene sequencing.

ARDRA results (Fig 2 and Fig 3) revealed that some of the nitrogen-fixing strains tested were similar, or identical, to reference strains described in the literature. ARDRA groups (Table 1); defined by similarities in ARDRA patterns were comprised by 17 clusters.

ARDRA is commonly utilized as an alternative to more laborious and expensive methods for the identification of eubacteria, being the analysis of the rRNA cistron is a good criterion for microbial classification at both genus and species level (Grimont and Grimont, 1986; Massol-Deya et.al 1995). Other studies have shown that more than one enzyme are necessary to resolve the 16S r-RNA gene of different species (Tchan, 1984; Moyer *et al.*, 1996). The significance of 16S rRNA gene sequencing for Bacterial identification is suggested by Janda and Abott (2007). Thus the biphasic approach proposed proved to be suitable for the identification of members of the different genera at the species level and represents a contribution to the disclosure and study of the microbial diversity, also in view of biotechnological exploitation of free-living nitrogen-fixing bacteria.

16S rRNA sequencing

After ARDRA analysis all the isolates were distributed into 17 groups, out of which 08 groups were identified but for

Table 1: Genomic analysis of isolated free-living nitrogen-fixing bacteria.

ARDRA group	Strain	Source	ARDRA pattern	Identified by 16S rRNA gene sequencing
1	Vis 2	Rhizospheric soil of castor plant	Unique	*Azotobacter tropicalis*[b]
1	Idr 3	Rhizospheric soil of castor plant	Unique	*Azotobacter tropicalis*[b]
2	Dt3	Rhizospheric soil of cotton plant	Similar with Kd1 and Kh1	*Arthrobacter* sp.[a]
2	Kd 1	Rhizospheric soil of cotton plant	Similar with Dt3 and Kh1	*Arthrobacter* sp.[a]
2	Kh1	Rhizospheric soil of cotton plant	Similar with Dt3 and Kd1	*Arthrobacter* sp.[a]
3	Ptn 4	Rhizospheric soil of grass	Unique	*Variovorax* soil strain[b]
4	Ch4	Rhizospheric soil of castor plant	Unique	*Streptomyces thermocarboxydovorans*[b]
4	Sat 3	Rhizospheric soil of castor plant	Similar with Ch4	*Streptomyces thermocarboxydovorans*[b]
5	Am 5	Rhizospheric soil of wheat	Unique	Isolate similar with uncultured bacterium sp.
6	Rd1	Rhizospheric soil of pearl millet	Similar with	*Enterobacter* sp. [a]
7	Bch 3	Rhizospheric soil of pearl millet	Unique	*Nocardioides nitrophenolicus*[b]
8	Trd 3	Rhizospheric soil of wheat	Similar with Am1, Ch1and Rd3	*Bacillus* sp. [a]
8	Am 1	Rhizospheric soil of wheat	Similar with Trd3, Ch1and Rd3	*Bacillus* sp. [a]
8	Ch 1	Rhizospheric soil of wheat	Similar with Am1, Trd3 and Rd3	*Bacillus* sp. [a]
8	Rd 3	Rhizospheric soil of wheat	Similar with Am1, Ch1 and Trd3	*Bacillus* sp. [a]
9	Pr 2	Rhizospheric soil of pearl millet	Similar with Msn4, Bch4, Ha3 and Kh2	*Azotobacter vinelandii* [a]
9	Msn4	Rhizospheric soil of pearl millet	Similar with Pr2, Bch4, Ha3 and Kh2	*Azotobacter vinelandii* [a]
9	Bch 4	Rhizospheric soil of pearl millet	Similar with Msn4, Pr2, Ha3 and Kh2	*Azotobacter vinelandii* [a]
9	Ha 3	Rhizospheric soil of pearl millet	Similar with Msn4, Bch4, Pr2 and Kh2	*Azotobacter vinelandii* [a]
9	Kh 2	Rhizospheric soil of pearl millet	Similar with Msn4, Bch4, Ha3 and Pr2	*Azotobacter vinelandii* [a]
10	Pln 4	Rhizospheric soil of cotton plant	Unique	*Rhodococcus corynebacterioides*[b]
11	Am 3	Rhizospheric soil of castor plant	Unique	*Mycobacterium cosmeticum*[b]
12	Ptn2	Rhizospheric soil of castor plant	Similar with Rd2, Vis4 and Ch3	*Agrobacterium tumifaciens* [a]
12	Rd 2	Rhizospheric soil of castor plant	Similar with Ptn2, Vis4 and Ch3	*Agrobacterium tumifaciens* [a]
12	Vis 4	Rhizospheric soil of castor plant	Similar with Rd2, Ptn2 and Ch3	*Agrobacterium tumifaciens* [a]
12	Ch 3	Rhizosphericsoil of castor plant	Similar with Rd2, Vis4 and Ptn2	*Agrobacterium tumifaciens* [a]
13	Kd 2	Rhizospheric soil of cotton plant	Unique	*Microbacterium* sp. [b]
14	Ha 2	Rhizospheric soil of castor plant	Similar with Hmt2	*Azotobacter chrococcum* [a]
14	Hmt 2	Rhizospheric soil of castor plant	Similar with Ha2	*Azotobacter chrococcum* [a]
15	Hmt 4	Rhizospheric soil of castor plant	Unique	*Azotobacter salinestris* [b]
16	Idr 4	Rhizospheric soil of cotton plant	Unique	*Planococcus* sp. [b]
17	Trd2	Rhizospheric soil of pearl millet	Similar with Ch2 and Hmt1	*Pseudomonas aerogenosa* [a]
17	Ch2	Rhizospheric soil of pearl millet	Similar with Trd2 and Hmt1	*Pseudomonas aerogenosa* [a]
17	Hmt1	Rhizospheric soil of pearl millet	Similar with Ch2 and Trd2	*Pseudomonas aerogenosa* [a]

a: Identified using ARDRA profiling, b: Identified by using 16srDNA sequencing.

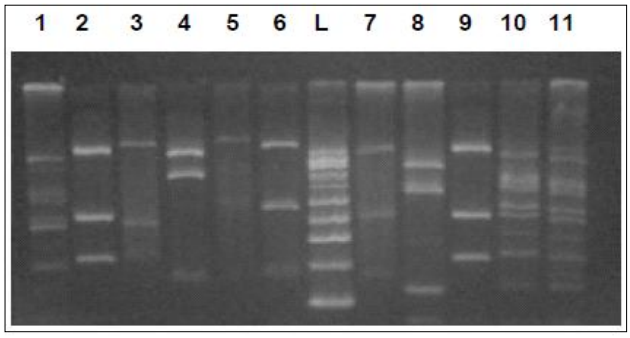

Fig 2: Agarose gel electrophoresis of 16srDNA digested with restriction enzyme Taq 1: Lane1 Vis; Lane 2 Dt 3; Lane 3 Ptn 4; lane 4-Ch4;Lane 5-AM 5; Lane 6- Rd 1; L- 100bp DNA Ladder; lane 7-Pln 4; Lane 8- Bch 3; Lane 9- Reference strain *Arthrobacter*; lane 10- Reference strain *Bacillus* sp.; lane 11- Trd 3.

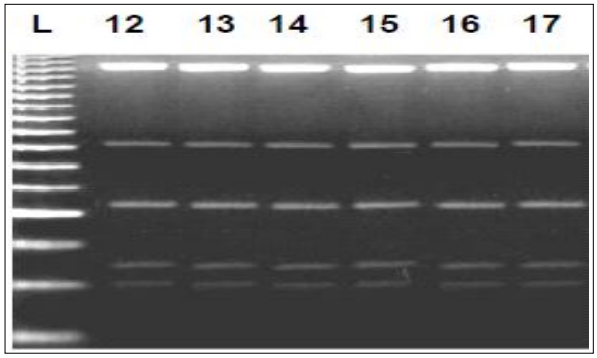

Fig 3: Agarose gel electrophoresis of 16srDNA digested with restriction enzyme *Hpa* II: L-200bP Ladder; Lane 12-Reference strain *A.vinelandii;* lane 13- Msn4; Lane 14-Pr2; lane 15- Bch4; lane 16- Ha 3; lane 17-Kh2.

the confirmation and identification up to species level representative strains from each group were analyzed for 16S r RNA gene sequencing. DNA sequences were compared with already submitted sequences in nucleotide databases available at NCBI website using BLAST software. The identification of isolates was carried out through BLAST analysis and sequences submitted to Genbank, NCBI. The accession number provided from Genbank are JX437935, JN591767, JX564632, JX564633, JN580892, JX564634, JX564635, KF418747, KF418748, KF418749, KF418750, KF535156, KJ538560, KJ538561 and KJ538562. 16S r-RNA Sequencing analysis one strain Am 5 is found to be similar with uncultured bacterium sp. So, the isolate Am 5 is regarded as unknown isolate right now and further work will be carried out later on.

Phylogenetic relatedness

Most similar sequences were aligned by ClustalW software and a phylogenetic tree was drawn to analyze evolutionary relationships among sequences of isolated microorganisms and nearest neighbors. The phylogenetic tree of isolate Hmt4 is shown in Fig 4. The position of isolate Hmt 4 in the phylogenetic tree is closely related with *Azotobacter salinestris* I-A strain. The phylogenetic tree of isolate Am 5 is shown in Fig 5. The position of isolate Am 5 indicated in the phylogenetic tree is closely related with uncultured bacterium ncd2510b01c1.

Strain Hmt4 was identified as an *Azotobacter salinestiss*. The potential of *Azotobacter salinestris* as Plant Growth Promoting Rhizobacteria under saline stress conditions was investigated (Abdel Latef *et al* 2021). The strain was also further confirmed by phenotypic data. The phylogenetic

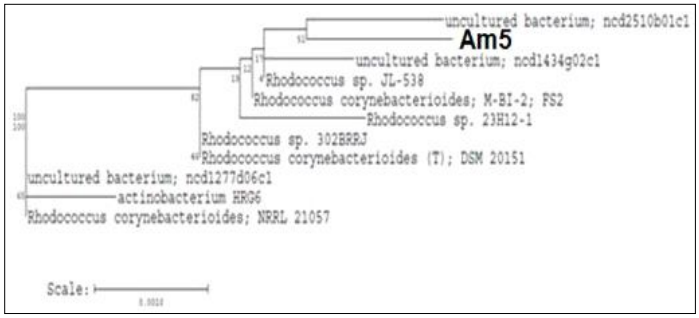

Fig 4: Phylogenetic analysis of strain Am5.

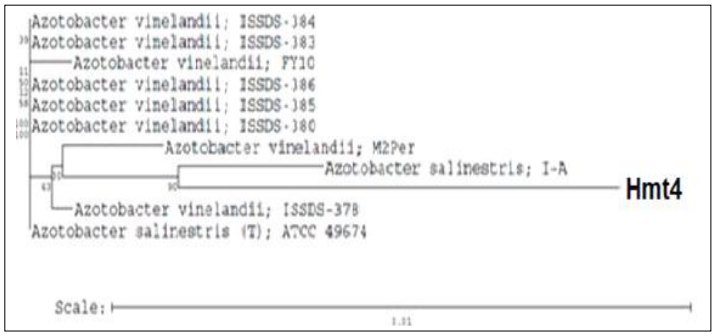

Fig 5: Phylogenetic analysis of strain Hmt4.

diversity of nitrogen fixing bacteria in the coastal waters of the South Eastern Arabian sea was suggested by (Jabir *et al.* 2018). *Agrobacterium tumefaciens* as associative nitrogen-fixing bacteria was suggested by My *et al.* (2015).

CONCLUSION

The use of chemical fertilizer and chemical pesticides in crop fields makes soil infertile. The demand of organic Crop in India and abroad is highly increasing day by day. In the present study free living nitrogen fixing bacteria have been characterized and identified at species level by using molecular characterization. A diverse group of bacteria has been identified from North Gujarat soil. The right use of isolated bacteria in the soil will improve the fertility and improve crop productivity.

Conflict of interest: None.

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