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DIVERSITY OF NON-SYMBIOTIC NITROGEN FIXING BACTERIA FROM SOILS OF NORTH GUJARAT, INDIA

[1]*P. Patel and [2]S. Bhatt

[1]Department of Biotechnology, Mehsana Urban Institute of Sciences, Mehsana-Gozariya Highway, Ganpat University-384012, Gujarat, India.
[2]Department of Life Sciences, Hemchandracharya North Gujarat University, Patan-384265, Gujarat, India.

***Corresponding Author: Dr. P. Patel**
Department of Biotechnology, Mehsana Urban Institute of Sciences, Mehsana-Gozariya Highway, Ganpat University-384012, Gujarat, India.

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ABSTRACT
Diversity of non-symbiotic nitrogen fixing bacteria isolated from four districts of North Gujarat; Mehsana, Sabarkantha, Banaskantha and Patan have been investigated. Non-Symbiotic nitrogen fixing bacteria were randomly selected for studying their Morphological and Physiological characterization, Biochemical Characterization, Genetic analysis for the identification up to species level, and Phylogenetic relatedness of the isolates. Total Fifty-nine strains of non-symbiotic nitrogen fixing bacteria were isolated from diverse agro-ecological locations of North Gujarat. DNA characterization by using Amplified ribosomal DNA restriction analysis could separate these bacteria into 16 different groups. They are found to be *Azotobacter salinestris, Azotobacter tropicalis, Azotobacter vinelandii, Nocardiodies nitrophenolicus, Streptomyces thermocarboxydovorans, Agrobacterium tumifaciens, one isolate similar with uncultured Sp., Variovorax soil strain, Mycobacterium cosmeticum, Enterobacter ludwigi, Rhodococcus* corynebacterioides, *Sinorhizobium saheli, Bacillus niabensis, Planococcus sp., Microbacterium sp., and Arthrobacter sp.* The genetic analysis showed that non-symbiotic nitrogen fixing bacteria are highly diverse in nature and cannot specific to any each ecosystem. Our result indicates to explore the Biotechnological applications in different fields of environmental microbiology along with their nitrogen fixing capacity.

KEYWORDS: *Nitrogen Fixing Bacteria, Amplified ribosomal DNA restriction,16S r-RNA, Diversity, Soil.*

INTRODUCTION
Symbiotic nitrogen fixing bacteria are the most significant biological Nitrogen source for many terrestrial ecosystems (Kennedy and Islam, 2001). However, non-symbiotic nitrogen-fixing microorganisms also contribute to the nitrogen demand of a number of ecosystems (Kahindi *et al.*, 1997; Deslippe *et al.*, 2005; Unkovich and Baldock, 2008; Hsu and Buckley, 2009). There are various genera of non- symbiotic nitrogen fixing bacteria found in soil include *Azotobacter*, *Beijerinckia* and *Derxia*, *Azospirillum*, *Klebsiella*, *Clostridium* and *Erwinia*. (Giller and Day, 1985). The nitrogen fixing ability of free-living nitrogen fixing bacteria in the field is strongly influenced by the prevailing environmental condition. They are especially sensitive to various environmental stresses which occur in the tropics can be divided into predominantly physical factor (temperature, moisture) and into chemical factors which include both toxic effects like acidity and nutrient deficiencies (McGrath *et al.*, 1995; Alexander and Zuberer, 1989; Tan *et al.*, 2003; Zhang *et al.*, 2006).

Prokaryotic organisms are difficult to classify, and the validity of the classification has been often questioned. The morphological characteristics such as cell

shape, cell wall, movement, flagella, Gram staining, etc. may not be adequate for establishing a detailed classification of microbes. Advances in molecular and chemical ecology have provided a promising alternative in estimating microbial diversity. Methods to measure microbial diversity in soil can be categorized into two groups, *i.e.*, biochemical techniques and molecular techniques (Amann *et al.*, 1995).

The molecular techniques includes DNA re-association, DNA– DNA and mRNA-DNA hybridization, DNA cloning and sequencing and other PCR-based methods such as Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), Ribosomal Intergenic Spacer Analysis (RISA) and Automated Ribosomal Intergenic Spacer Analysis (ARISA), Ribosomal DNA Restriction Analysis (ARDRA) (Kirk *et al.*, 2004). The sequence based techniques of molecular biology can be applied to the

study of natural microbial ecosystems. These methods characterize the microbial processes and thereby can be used to reach a better understanding of microbial diversity. These techniques can be used to quantitatively analyze microbial diversity and expand our understanding of their ecological processes. All these methods justify the interest recently arisen on the development of analytical tools suitable for a rapid and reliable identification of soil bacteria and for their study in soil microbial communities.

In this context, the Conventional biochemical methods and the potential molecular methods for e.g. Amplified Ribosomal DNA Restriction Analysis (ARDRA) and 16S rRNA sequencing for the identification, characterization and differentiation of various free living nitrogen fixing bacteria were evaluated. This approach allowed a sound method for the unambiguous identification of free living nitrogen fixers to be developed, also in view of deepening knowledge on the ecology of these microorganisms and their biotechnological exploitations.

The objectives of the research is to investigate diversity of non-symbiotic nitrogen fixing bacteria isolated from North Gujarat region with regards to diversification in genetic level and in conjunction with population changes in diverse ecosystems. This might be able to bring about the management for proper utilization of free-living nitrogen fixing bacteria for increasing soil fertility in sustainable agriculture and forestry.

MATERIALS AND METHODS

Study area

The major topographic research locations selected for study are four districts of North Gujarat viz., Sabarkantha, Mehsana, Banaskantha and Patan. Total five towns were selected from each district and from each town place five different sampling sites were selected. Mean annual rainfall in North Gujarat according to the records of the Indian Meteorological Department is 700 mm, of which more than 95% is received during the monsoon season. The rains are highly erratic in both intensity and duration. Sometimes, the intervals between the two successive rains may extend more than 20 days. Total numbers of rainy days are thirty to thirty five. The post monsoon period is generally hot and air temperature is generally higher during this period. Winter is mild with mean maximum temperature of 25℃ and mean minimum of 15 ℃. Summer is hot and dry. During the month of May, the mean maximum temperature goes up to 43 ℃ and mean minimum temperature reaches 28 ℃. There is no moisture surplus throughout the year and there is consistent moisture deficiency excet in the months of J uly and August. The underlying rock at Sabarkantha and Banaskantha districts in North Gujarat is basalt. In situ weathering leads to fragmentation of rocks by spheroid weathering of concentric rings. The soil derived from the rocks can be divided in to three basic types; viz; (1) 'Morrrum' on hill tops and exposed plateau, (2) Thin sandy clayey loam intermixed with pebbles on slopes and (3) Clayey black cotton soil in the low lying areas and plains which are highly sticky and form characteristic cracks upon drying. At the research site the soil varies in thickness from 15 cm to 1 m. At several places, rocks are exposed. Uppermost layer of the rock is amygdaloidal with vesicular cavities, filled with secondary deposits of quartzite, calcite, zeolite, agate etc. Sometimes, the metamorphosed instrugens may be 10 cm in diameter. The most prevalent color of the rock is grayish green.

Physical and Chemical analysis of Soil

The soil samples were dried at room temperature and then passed through 1 mm sieve prior to oven dry at 105 °C. These soil samples were used for chemical analysis as follows:

Soil pH was estimated by making soil paste in distilled water in ratio of 1:5 and measured on a Digital pH meter using glass electrode (Systronic make, model- 335). Soil organic carbon was estimated by wet oxidation method (Modified Walkely Black method) as per Makeague (1978). The organic matter was calculated by multiplying organic carbon by the factor 1.72 as per Richards (1968). Total soil nitrogen was determined by micro-kjedahl method (Peach and Tracey, 1956). Soil phosphorous was estimated by phosphomolybdate blue color method (Jackson, 1973). Exchangeable Na, K, Ca, and Mg of the soil samples were estimated on atomic absorption spectrophotometer (Varian Tech ron make, AA-6D).

Isolation of Free-living nitrogen fixing bacteria

The soil sample collected from each site was enriched in N_2 free medium (Ashby's Mannitol broth) and the non-symbiotic bacterial colonies were isolated on N_2 free agar medium (Ashby's Mannitol Agar medium). Total 59 strains were preserved at low temperature for further analysis.

Molecular analysis

DNA Isolation and purification

The freshly grown culture was centrifuged and the sediment, after washing, was resuspended in 500 µl of lysis buffer (10 mM Tris-HCl, 5mM EDTA, pH 7.8) with lysozyme (3 mg/ml). After incubation at 37°C for 1 h, 12.5 µl SDS (20%) and 5 µl proteinase K (10 mg/ml) were added and the mixture was incubated at room temperature for 16 hours. DNA was isolated from the crude lysates of cells by phenol extraction (Sambrock & Russell 2001) and resuspended in 50 µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.8). The obtained DNA was quantified by UV spectrum (260 nm) and DNA integrity was verified by 0.8% agarose gel electrophoresis.

ARDRA (Amplified Ribosomal DNA Restriction Analysis)

In order to choose the restriction enzymes to be used for

ARDRA, the 16S rRNA sequences of different nitrogen fixing bacteria retrieved from the GenBank were virtually restricted using the serial cloner software (Version 2.6). Crude DNA was isolated from bacterial culture using Chromous Bacterial genomic DNA Isolation Kit RKN. The 16S r-RNA gene was amplified by means of universal 16S r-DNA primers and the reactions were run for 35 cycles. The product obtained (1.5kb) was digested with TaqI, HpaII and RsaI (4 base cutters). Five units of each enzyme were added to 12.5 µl of the amplification product and incubated for 5 h at 37 °C in a total volume of 25 µl. The digests were resolved by electrophoresis on a 2% agarose gel along with 100bp and 250bp DNA ladder. Gels were stained in ethidium bromide for 15 min and rinsed for 5 min in distilled water. Gel electronic images were visualized to check the ARDRA pattern. The ARDRA pattern of all the isolates was compared with the ARDRA profile of different nitrogen fixing known reference strain of bacteria. The comparison of amplified DNA profiles was performed on the basis of the presence (1) or absence (0) of fragments was generated by using PyElph 1.4 software. 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 dandrogram was generated in Newick format (Fig.1).

PCR-Sequencing

After purification of the PCR product it was sent for sequencing to Chromus Biotech pvt. Ltd., India. The sequencing of the purified products was done as follows. The sequencing mix composition and PCR conditions used is described later. Sequencing Machine used was ABI 3500 XL Genetic Analyzer and the chemicals for chemistry used were: Big Dye Terminator version 3.1" Cycle sequencing kit. For Polymer & Capillary Array: POP_7 polymer was used for 50 cm Capillary Array. The results were analyzed by using protocol BDTv3-KB-Denovo_v 5.2. Data analysis was carried out by Seq Scape_ v 5.2 Software and the reaction plates used in the sequencing were Applied Biosystem Micro Amp Optical 96- Well Reaction plates. The partial rRNA gene sequences of the isolates were preliminarily compared with those present in the Basic BLAST search (Altschul *et al.*, 1997), and then aligned using the Clustal W program (Higgins *et al.*, 1992) available at the European Bioinformatic 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/).

RESULTS

The Soils

The recorded soil temperature showed inverse relationship with the soil depth. Soil moisture was found maximum during the monsoon months and it gradually decreased in the subsequent months, however, it was little more at 30 cm soil depth. There was some marked difference in soil texture, and colour in various sites (Table 1). Site of Patan was having pH in range of 6.5 to 7.46, while sites of Banaskantha and Sabarkantha were having pH in range of 7.11 to 7.67 and the sites of Mehsana were having pH in range of 7.07 to 7.62. Soil chemical characters show that there was not much variations in their characters, however, soils of Patan district contained little more organic carbon and total nitrogen than the soils at the other districts (Table 2).

Table 1: Physical properties of the soil.

Name of site	Coarse sand (%)		Fine sand (%)		Silt and clay (%)		Soil temperature °C		Soil Colour
	Min	Max	Min	Max	Min	Max	Min	Max	
Sabarkantha	42	58	38	46	20	31	17.6	36.8	Brown
Mehsana	37	45	30	45	33	47	13.6	37.5	Grayish Brown
Banskantha	32	46	34	51	34	45	19.2	38.6	Brown
Patan	33	43	43	51	24	32	11.4	40.3	Brown

Table 2: Chemical properties of the soil.

Sr. No	Name of Site	pH	Total Nitrogen (mg/L)	Organic Carbon (%)	Available Phosphorus (%)	Ca Hardness	Mg Hardness	Na^+ ppm	K^+ ppm
1	Mehsana	7.07-7.62±0.004	11.2±0.0021	0.43±0.063	0.05±0.006	0.82±0.005	0.07±0.002	65.7	55.2
2	Patan	6.5 -7.460.001	18.48±0.004	0.76±0.01	0.08±0.004	0.04±0.001	0.028±0.001	71.4	16.8
3	Banaskantha	7.11- 7.67 ±0.003	13.2±0.002	0.31±0.02	0.065±0.005	0.050±0.005	0.026±0.003	65.9	61.3
4	Sabarkantha	7.11- 7.67 ± 0.05	65.4±0.05	0.42±0.05	0.06±0.002	0.033±0.001	0.005±0.001	25.7	48.1

± Standard deviation

Amplified restriction digestion analysis

Stringent PCR conditions allowed amplification of a single 16S r-DNA fragment. All isolates yielded a band of ~1.5kb in size after amplification with the universal eubacterial primer. ARDRA (Amplified restriction digestion analysis) of extracted DNA of each bacteria were digested with TaqI, HpaII and RsaI (4 base cutters) resulted in the number of pattern type (Fig. 1 to Fig. 3). The isolates were grouped into different 16 cluster types. From 16 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 r-RNA sequencing.

Cluster Analysis

A total of 59 non-symbiotic nitrogen fixing bacterial DNA band positions could be differentiated in ARDRA analysis. All the bands were also retrieved, followed by PCR, cloning and sequencing. As a result, total 16 different types of free living bacterial species were identified up to species level. Using Jaccard's genetic similarity coefficient, we established that different levels of genetic variation exist among the isolates, ranging from 0.013 to 0.33. The UPGMA cluster analysis of the Jaccard's similarity coefficient was used to generate a dendrogram (Fig.4), illustrating the overall genetic relationship among the free living nitrogen fixing bacteria. According to this analysis, 59 free living nitrogen fixing bacteria were grouped into sixteen major clusters at a coefficient level of 0.001 (Table 3). A large variation was observed among the free living nitrogen fixing bacteria, which could be due to the different soil types within the study area.

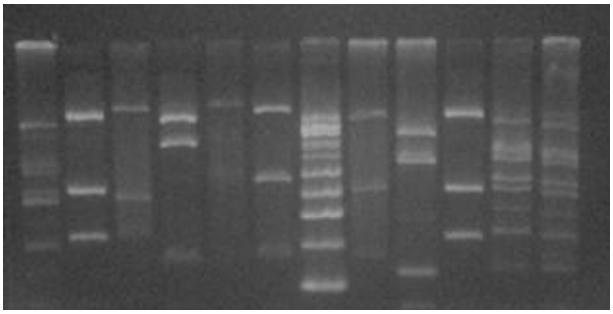

Fig. 1. ARDRA profile of selected strain after digestion *with* Taq 1.

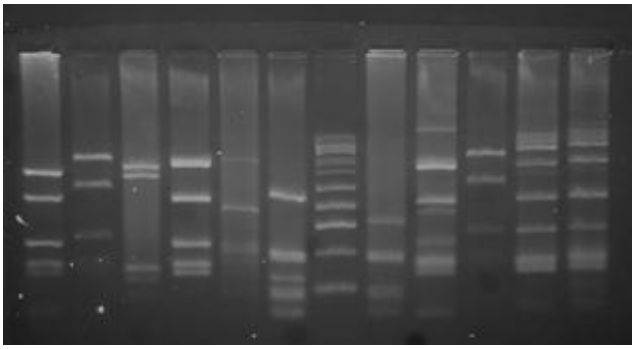

Fig. 2 ARDRA profile of selected strain after digestion with *HpaII.*

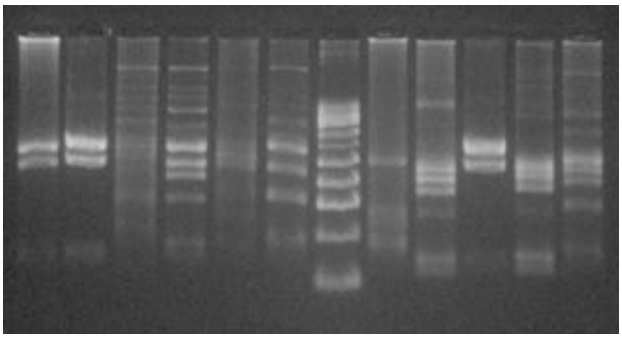

Fig. 3. ARDRA profile of selected strain after digestion *with RsaI.*

Table 3: Cluster analysis and identification of the isolated free living nitrogen fixing bacteria.

Cluster	Isolate name	No. of isolates	Identified as	Accession number
1	Vis 2, Idr 3, Dt 1, Bch 2	4	*Azotobacter tropicalis*	JN591767
2	Dt3, Kd 1, Kh1	3	*Arthrobacter sp.*	KJ538562
3	Ptn 4, Mgh 1	2	*Variovorax soil strain*	JX564635
4	Ch4, Sat 3, Idr 1, Ha 1, Bch 1	5	*Streptomyces thermocarboxydovorans*	JN580892
5	Am 5	1	Isolate similar with Uncultured bacteriumsp.	--
6	Rd1, Mgh2, Vij 3	3	*Enterobacter ludwigi*	KF418748
7	Bch 3	1	*Nocardioides nitrophenolicus*	JX564633
8	Trd 3, Am 1, Ch 1, Rd 3	4	*Bacillus niabensis*	KF535156
9	Msn4, Bch 4, Ha 3, Kh 2, Pr 2, Trd 1, Vis 1, Sat 2, Dt 2	9	*Azotobacter vinelandii*	JX564632
10	Pln 4	1	*Rhodococcus* corynebacterioides	KF418749
11	Am 3	1	*Mycobacterium cosmeticum*	KF418747
12	Vij 2, Rd 4	2	*Sinorhizobium saheli*	KF418750
13	Ptn2, Rd 2, Vis 4, Ch 3, Hmt 3, Kh 3, Pr 3, Vij 4, Am 2, Idr 2, Am4, Ptn 1	12	*Agrobacterium tumifaciens*	JX564634
14	Kd 2	1	*Microbacterium sp.*	KJ538561
15	Hmt 4, Ha 2, Hmt 2, Msn 1, Vis 3	1	*Azotobacter salinestris*	JX437935
16	Idr 4, Trd2, Ch2, Hmt1, Ptn3	5	*Planococcus sp.*	KJ538560

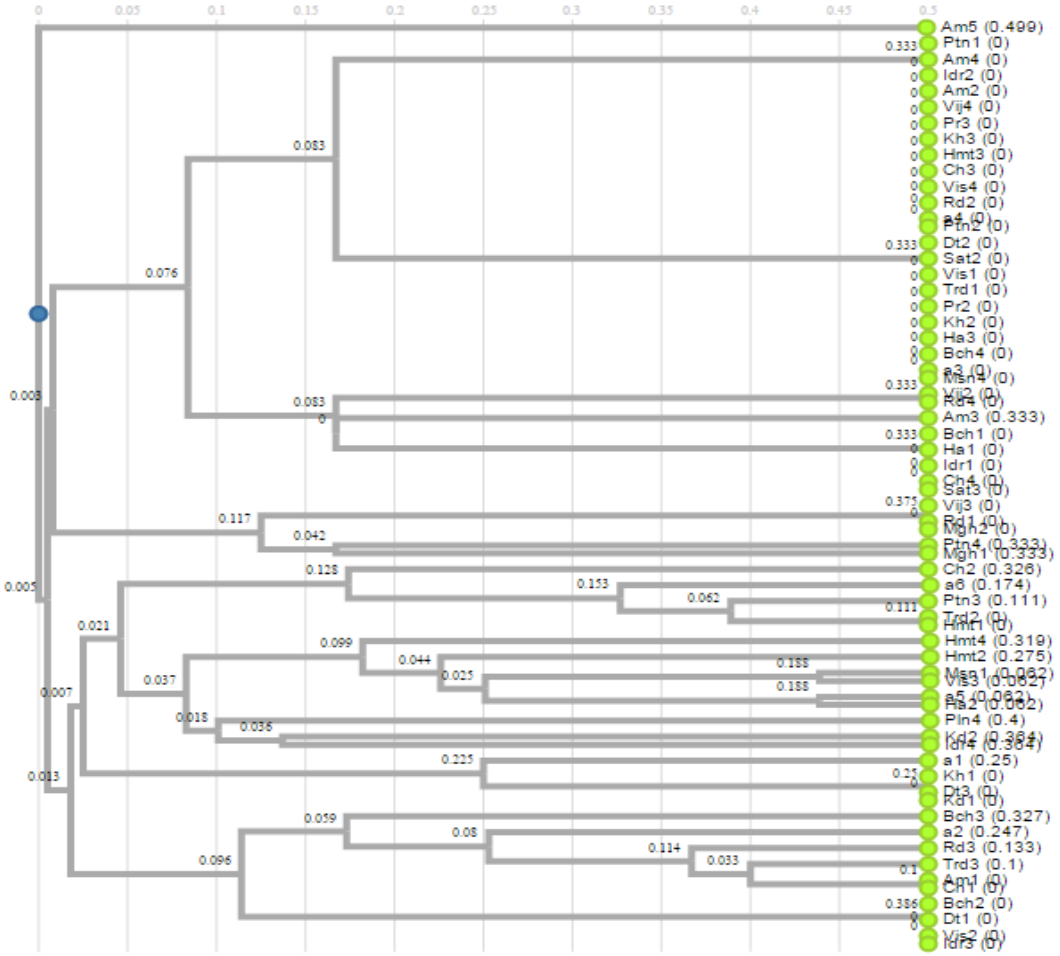

Fig. 4 UPGMA dendrogram of all the isolates taken under study including five reference strains digested with Taq I.

DISCUSSION

Successful isolation of the non-symbiotic nitrogen fixing bacteria from different region of North Gujarat are able to grow on N_2 free medium, suggested that all the 59 isolates of bacteria are free living Nitrogen fixing bacteria. Nitrogen fixing microorganisms are highly adapted to different environmental conditions and considered to be important for the nitrogen input to soil; they are rarely dominant in terrestrial ecosystems (Wartiainen et al. 2008; Coelho et al. 2009) and susceptible to environmental condition. In this study, the diversity of non-symbiotic nitrogen fixing microbial community in North Gujarat region is varied with the physico-chemical properties of soil, plant species, and the development period of plant community, and was influenced by plant rhizosphere. In the North Gujarat region the soil types where more vegetation is found, recorded more of free living nitrogen fixing bacteria few of which have been found to be potential N_2 fixers and those can be further tested to be used as Biofertilizer in the soils of same districts as well as others. The morphological and cultural characteristics found that out of 59 free-living nitrogen fixing bacterial isolates, majority of the strains are gram negative short rods, some of the strains are gram positive rods and filamentous and some are gram positive cocci (Data not shown). The result was agreed with Chocnhahirum (1986) who found that 259 bacterial cultures isolated from acid soil of Thailand were gram negative rod shape.

The diversity of free-living diazotrophs depends on several soil environmental factors, such as the soil pH, C abundance and N availability (Hsu SF *et. al.*).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 a good criterion for microbial classification at both genus and species level (Grimont and Grimont, 1986; Massol-Deya *et al.*, 1995). However, this molecular tool has not been utilized on Azotobacteraceae and other nitrogen fixing bacteria whose identification, traditionally based on conventional biochemical tests, is often tentative. Therefore, the development of a simple identification method yielding reliable and unambiguous results appears to be useful. The first step in developing this method was the selection of the enzymes to be used for the 16S r-RNA gene restriction analysis. In order to avoid time consuming and low-yielding experimental work, the sequences of Azotobacteraceae and other commonly found nitrogen fixing bacteria, available in

ribosomal databases, were subjected to computer analysis of digestion sites. 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). Three enzymes, namely, HpaII, RsaI, and TaqI were selected for digestion. Results showed that the use of this enzyme on reference strains leads to the clear discriminate between the different Azotobacteraceae species as well as some other nitrogen fixing bacteria. This is in agreement with the results obtained by Tiedje *et al.* (1999) which evidenced that HhaI, RsaI, and HpaII gave the greatest resolution in terms of philogenetical dissection of soil natural communities, and by Moyer *et al*. (1996) who described HhaI and RsaI as optimal for the detection and differentiation of bacterial taxa, on the basis of the average number of restriction sites. Isolates formed various clusters with the various biochemical properties. The diazotrophs were found to differ in the total genetic diversity and most of the non-symbiotic bacteria followed the same clustering groups like cluster analysis with little exception in clusters (Rafii *et. al*).

CONCLUSION

The Non-symbiotic nitrogen fixing bacterial strain from soils of North Gujarat is highly diverse in terms of species. They are not specific related with each ecosystem. The tropical soils of North Gujarat, India mainly dominated by β-subclass of Proteobacteria: 12 non-symbiotic bacteria were identified as an *Agrobacterim tumifaciens* and 09 isolates were identified as an *Azotobacter vinelandii.* One of the isolate sequence was closely related to uncultured microorganisms which may represent novel sequences of nitrogen-fixing microorganism. This work is further to be extended to explore the Biotechnological applications for improving nitrogen fixing capacity.

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