

## Amplified Ribosomal DNA Restriction Analysis for the Preliminary Characterization of Native Diazotrophs *Azotobacter*, *Acetobacter* and *Azospirillum*

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A 16S rRNA gene-based fingerprinting method was developed for the preliminary characterization of native diazotrophs bacterial isolates *Azotobacter*, *Acetobacter* and *Azospirillum* tested with reference strains belonging to the free-living nitrogen-fixing bacterial group. According to this method, the 16S rRNA gene was amplified using universal primers and PCR products were subsequently digested with *TaqI*, *BamHI*, *HinfI* and *HaeIII*. The analysis of the restriction profiles obtained showed that the method is able to define a unique species-specific phylotype (SSP) for each of the native diazotrophic bacterial isolates. Phylogenetic analysis of 16S rRNA indicated that these diazotrophic bacterial isolates belonged to respective genus *Azotobacter*, *Acetobacter* and *Azospirillum* and closely related to standard strains.

**Keywords:** Diazotrophs, 16s rRNA, Restriction enzyme, *Azotobacter*, *Acetobacter*, *Azospirillum*.

Nitrogen is a major constituent of chlorophyll, proteins, nucleic acid and responsible for many more functions in a plant. Therefore, it is considered as a major nutrient required for crop growth and productivity. There are basically four known processes to convert atmospheric nitrogen to forms, which can readily be absorbed and utilized by plants: 1) Atmospheric Nitrogen Fixation, 2) Industrial Nitrogen Fixation, 3) Volcanic Expulsion and 4) Biological Nitrogen Fixation (BNF). The demand for higher crop production also implies a higher demand for fixed nitrogen.

Chemically produced nitrogenous fertilizers can provide fixed nitrogen, but the production of fertilizer is expensive and also harmful

to the environment. Its damage includes change in nitrogen cycle, loss of nitrous oxides to the atmosphere, acid rain, nitrate pollution of ground water and induced leaching of soil nutrients. The BNF system in bacteria increases the potential nitrogen supply by fixing nitrogen directly with little or no loss. An inexpensive and eco-friendly alternative to reduce usage of nitrogenous fertilizer is BNF. It is done by Nitrogen-fixing bacteria (Diazotrophs means *di*: two, *azo*: nitrogen, *trophs*: lovers). BNF is estimated to contribute 1.8x10<sup>8</sup> metric tons of nitrogen globally, every year of which eighty per cent is by symbiotic bacteria and the rest from free living and associative systems (Graham, 1988). The ability of such appreciable amounts of nitrogen from the atmospheric reservoir to enrich the soil is the key role of bacteria.

Bacterial colonization of internal tissues of healthy plants is a common phenomenon by

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several species of bacteria on a wide range of host plant species (Azevedo, 1998). Microaerophilic bacteria, such as *Azospirillum* spp., *Burkholderia* spp. and *Herbaspirillum* spp. colonize roots, shoots and leaves of maize, bajra, rice and wheat (Dobereiner, 1989). Other facultative anaerobic species, including *Citrobacter*, *Enterobacter*, *Erwinia* and *Klebsiella*, may establish associations with grasses and few strains are able to fix nitrogen (Eady, 1992). All such important bacteria are having specific characteristics and described here after; with molecular approaches for their characterization. BNF is a microbiological conversion of  $N_2$  into ammonia with the help of nitrogenase enzyme present in the bacteria of genus *Azotobacter*, *Acetobacter*, *Azospirillum*, (non symbiotic and associative); as well as by *Rhizobium* (symbiotic) etc. Nitrogen-fixing bacteria are able to fix atmospheric nitrogen under different conditions in association or in strict symbiosis. Diazotrophs are found in a wide variety of habitats including free-living in soil and water, associative symbiosis with grasses, actinorhizal associations with woody plants, aquatic ferns associated with various plants and root-nodule symbiosis with legumes. Bacteria convert atmospheric ( $N_2$ ) to ammonia, which can be easily used by the plant and ultimately for its growth in a carbon-rich and N-poor environment. Diazotrophs can be considered as plant growth promoting rhizobacteria (PGPR), a group of bacteria which directly play many beneficial effects on crops to uplift the yield (Vande Broek and Vanderleyden, 1995; Dobbelaere et al., 2003). In addition to reducing the need for nitrogenous fertilizers (Dawe, 2000), the diazotrophic associations with plants increases the efficiency of the applied chemical fertilizers (Okon and Labandera-Gonzalez, 1994; Kennedy et al., 1997; Gunarto et al., 1999). Although the magnitude of BNF from biofertilizer may account for a 30 to 40 % of total N requirement of crop and, reduces losses in ecosystem with significantly contributing to N economy for crop production (Kennedy et al., 2004).

In context of identification and characterization of various *Azotobacter* spp., such as selected media, phynotypical, biochemical characteristic give inconsistency in proper identification on other hand molecular marker can be successfully employed for such categorization

(Knowles, 1982). Molecular approaches have been developed and successfully applied to describe such diazotrophic communities in different cultivated and forest soils (Poly et al., 2001 and Shaffer et al., 2000), pasture, agricultural soils (Poly et al., 2001), wetland soils (Chelius, 1999) and rhizospheres (Hamelin et al., 2002) among various molecular markers technique restriction enzyme based method have been use successfully many year (Jayarao et al., 1992 and Aquilanti, et al., 2004).

Amplified Ribosomal DNA Restriction Analysis (ARDRA) is the extension of the RFLP (restriction fragment length polymorphism) technique encoding the small (16s) ribosomal subunit of bacteria. ARDRA has proved to be useful for the differentiation of bacterial strains at different taxonomic levels, depending on selection of conserved or variable regions followed by digestion using tetracutter restriction enzymes in the ribosomal genes. Patterns obtained from several restriction enzymes can be used for the phylogenetical characterization of cultured isolates (Swings, 1996; Tiedje, 1996).

Looking at the important of diazotrophs and their identification among other diazotrophic species from same environment. Stringent or a powerful tool is required to identification and characterization of native diazotrophs their for the present investigation is design to employ ARDRA, A powerful molecular tool to identify and characterized native diazotrophs and its similarity with different reference strains bearing biological nitrogen fixer potently.

## MATERIALS AND METHODS

### Bacterial strain and Cultures revival

The native diazotrophic bacterial isolates and four reference strains grown in respective nitrogen free media viz, Burk's medium for *Azotobacter*, diluted cane juice semi solid medium for *Acetobacter* and NFB (Nitrogen Free Bromothymol blue medium) for *Azospirillum* (Cavalcant and Dobereiner, 1989) Table-1.

### 16S rRNA Amplification and Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Genomic DNA of all native diazotrophic bacterial isolates and standard strains were isolated using the protocol described by Sambrook et al. (1989). The amplification of the 16S rRNA gene by

PCR was performed in PCR reaction mixture (25  $\mu$ l) containing 2.5  $\mu$ l Taq Buffer (10 X), 0.5  $\mu$ l dNTPs (2.5 mM each) mix, 2.0  $\mu$ l Template DNA (25 ng/ $\mu$ l), 0.2  $\mu$ l Taq polymerase (5U/ $\mu$ l), 17.8  $\mu$ l Millipore Sterilized Water using the following primer 1.0  $\mu$ l Primer 1 (27 F-5'-AGAGTTTGATCC TGG CTC AG-3') and 1.0  $\mu$ l Primer 2 (1492 R-5'-GGT TAC CTT GTT ACG ACT T-3') and the primers synthesized at MWG Bio-tech Pvt. Ltd., Germany. These primers designed on the basis of conserved sequences of eubacteria (Weisburg *et al.*, 1991), were located at the extreme 50 and 30 of the 16S rRNA gene, respectively, allowing an approximately 1500-bp DNA fragment to be amplified. After mixing of all the components polymerize chain reaction was carried out in Mastercycler Personal (Eppendorf, Germany) with initial denaturation step at 94 C for 5 min followed by 35 cycles of denaturation (94 C for 1 min), annealing (58 C for 1 min) and extension (72 C for 2 min) and final extension step at 72 C for 10 min. PCR amplified

products were run on agarose gel electrophoresis.

16S rRNA gene PCR product (10  $\mu$ l) of each native diazotrophic bacterial isolates was used to carry out the restriction digestion with four different Tetra-cutter restriction enzymes (*TaqI*, *BamHI*, *HinfI* and *Hae III*). The reaction condition and components are described in Table 2.

The digested products were visualized and documented in gel documented system (Alpha innotech)

#### Statistical analyses of ARDRA patterns

Relationships between the native diazotrophs were established by using data from restriction analysis that adequately differentiated native isolates. A binary scoring system (1 for presence of band and 0 for the absence) was used to generate input matrix, which was analyzed using the unweighted pair group method using average (UPGMA) algorithm (Sneath and Sokal, 1973); a dendrogram was generated from the matrix using NTSYS pc software (Abaidoo *et al.*, 2002).

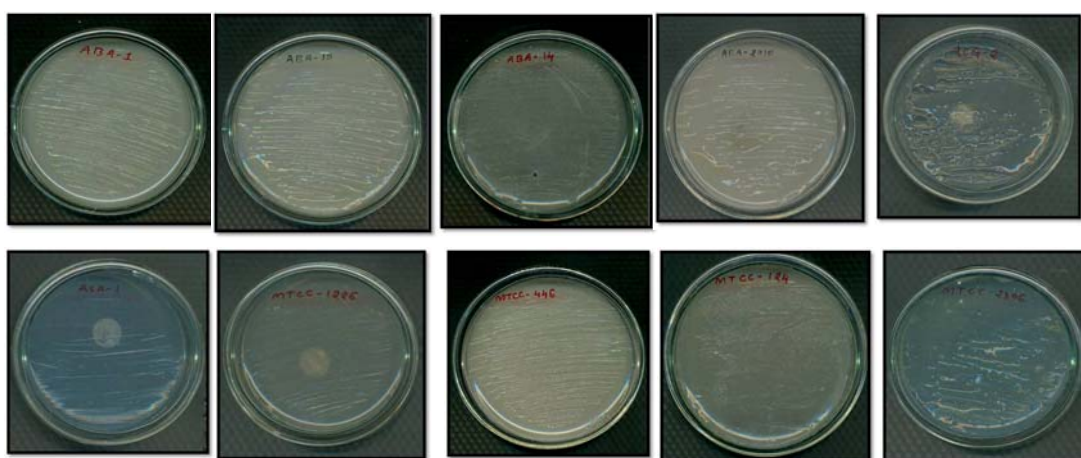
**Table 1.** Composition of different media used for strain isolation and revived of native diazotrophs.

Ingredient	Burk's medium gm/lit.	Nitrogen Free Bromothymol blue medium gm/lit.	LGIP medium gm/lit.
Malic acid	-	5 g	-
KOH	-	4 g	-
K <sub>2</sub> HPO <sub>4</sub>	0.8 g	-	0.2 g
KH <sub>2</sub> PO <sub>4</sub>	0.2 g	0.5 g	0.6 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.2 g	0.01 g	0.2 g
NaCl	0.2 g	0.02 g	-
CaSO <sub>4</sub>	0.1 g	-	-
CaCl <sub>3</sub>	-	-	0.02 g
H <sub>3</sub> BO <sub>3</sub>	100 mg	-	-
FeCl <sub>3</sub>	-	-	0.01 g
ZnSO <sub>4</sub> .7H <sub>2</sub> O	100 mg	-	-
FeSO <sub>4</sub> .7H <sub>2</sub> O	-	0.05 g	-
MnSO <sub>4</sub> .4H <sub>2</sub> O	10 mg	0.01 g	-
CuSO <sub>4</sub> .5H <sub>2</sub> O	3 mg	-	-
Na <sub>2</sub> MoO <sub>4</sub>	-	0.002 g	0.002 g
Cane sugar	-	-	100 g
KI	1 mg	-	-
FeMo mixture	1 ml	-	-
Bromothymol blue (0.5% alc.soln )	-	2 ml	5 ml
Distilled water	1000 ml	1000 ml	1000 ml
Agar	20 g	1.75 g	20 g
pH	7.3	6.6-7.0	6.0
FeMo mixture: FeCl <sub>3</sub> .6H <sub>2</sub> O-14.5 g	-	-	-
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O-2.53 g	-	-	-

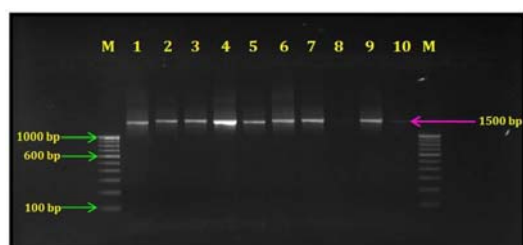
**Table 2.** Reaction Component and conditions for ARDRA.

Component/Condition	<i>Taq I</i> (5 U/ $\mu$ l)	<i>Bam H I</i> (5 U/ $\mu$ l)	<i>Hinf I</i> (5 U/ $\mu$ l)	<i>Hae III</i> (5 U/ $\mu$ l)
1. Reaction Component ( $\mu$ l)				
a. 10X assay buffer	2.00	2.00	2.00	2.00
b. Restriction enzyme	1.75	1.75	1.75	2.20
c. Nuclease free water	6.25	6.25	6.25	5.80
d. PCR product	10.00	10.00	10.00	10.00
Total Volume	20.00	20.00	20.00	20.00

2. Reaction Condition: All the reaction mixtures were incubated at 37 °C for 3 hrs and enzymes were deactivated at 65°C for 10 min after incubation.



**Fig. 1.** Diazotrophic native isolates (a) ABA-1, (b) ABA-10, (c) ABA-14, (d) ABA-2010, (e) ACG-2, (f) ASA-1 and reference strain (g) MTCC-446, (h) MTCC-124, (i) MTCC-1226 and (j) MTCC-2306 revive on their specific nitrogen free agar medium



**Fig. 2.** Agarose 1.8% gel electrophoresis of 16S rDNA amplification from diazotrophic isolates and respective reference strains using universal 16S rDNA primers. Lines 1-6, Azotobacter strains, 1. ABA-1, 2. ABA-10, 3. ABA-14, 4. ABA-2010, 5. MTCC-446 (reference strain), 6. MTCC-124 (reference strain), Lines 7 and 8, Acetobacter strains, 7. ACG-2 8. MTCC-1226 (reference strain), Lines 9 and 10, Azospirillum strains, 9. ASA-1 10. MTCC-2306 (reference strain)

## RESULTS AND DISCUSSION

### Bacterial strain and cultures revival

Six native diazotrophic bacterial isolates and four reference strains were revived (Fig. 1) and preserved on their specific and selective nitrogen free medium on recommended laboratory media like *Azotobacter* strains grown on Burk's media, *Acetobacter* strains grown on diluted cane juice semi solid medium and *Azospirillum* strains grown on nitrogen free bromo-thymol blue (NFB) media (Table 1).

### PCR and ARDRA products

Approximate products 1500 bp portion of the 16S rRNA gene from the diazotrophic isolates were amplified using the universal primers 27f and 1495r. They showed successful amplification of

**Table 3.** Amplified rDNA restriction analysis (ARDRA) pattern of native diazotrophic isolates obtained with enzyme *TaqI*.

Sr. No.	Diazotrophs isolates	Native diazotrophic isolates and standard strains	<i>TaqI</i>		ARDRA Pattern		<i>HaeIII</i>	
			Total no. of Bands	Restriction fragment size(bp)	Total no. of Bands	<i>HinfI</i> Restriction fragment size(bp)	Total no. of Bands	Restriction fragment size(bp)
1	<i>Azotobacter</i>	ABA-1	3	900, 311, 180	3	1000, 325, 168	5	435, 376, 310, 227, 182
2	"	ABA-10	4	515, 409, 352, 180	4	617, 369, 331, 168	4	609, 467, 310, 127
3	"	ABA-14	4	840, 283, 180, 110	4	1000, 206, 168, 128	4	540, 221, 182, 102
4	"	ABA-2010	4	695, 361, 180, 103	3	1000, 200, 128	4	502, 221, 182, 127
5	"	MTCC-446	3	875, 311, 180	3	1140, 168, 114	4	896, 221, 182, 127
6	"	MTCC-124	3	852, 279, 180	4	1000, 209, 168, 135	4	567, 221, 182, 102
7	<i>Acetobacter</i>	ACG-2	4	529, 420, 366, 180	4	650, 383, 331, 168	4	655, 490, 318, 127
8	"	MTCC-1226	4	529, 420, 366, 180	4	650, 383, 331, 168	4	671, 490, 318, 127
9	<i>Azospirillum</i>	ASA-1	3	924, 357, 180	3	1000, 337, 168	3	639, 310, 127
10	"	MTCC-2306	3	864, 357, 180	3	1000, 291, 168	4	502, 268, 182, 127

targeted amplicon in all diazotrophic isolates as well as reference strains (Fig. 2). Similar result were obtain by Aquilanti *et al.* (2004)

Products of 16S rRNA obtain from different isolates as well as reference strains were subjected to restriction digestion with *TaqI*, *BamHI*, *HinfI* and *HaeIII*. The digested products (Fig. 3) showed total of 110 fragments by 3 restriction enzyme *viz.*, *TaqI*, *HinfI* and *HaeIII* were as *BamHI* was unable to digested all samples (Fig. 3b). Number of restriction fragments obtained with each enzyme are shown in Table-3.

#### Fragment analysis

In order to assess the existence of species specify, restriction patterns were obtained for each of the enzyme utilized and have showed different restriction patterns for the diazotrophic isolates.

As reported (Table 2), the enzyme *TaqI* showed the restriction pattern of 16S rRNA gene of the diazotrophic isolates permitted to obtain 35 DNA restriction fragment. Restriction analysis on to diazotrophic isolates allowed four unique species specific restriction profiles obtained within the genus (Fig. 3a) While enzyme *HinfI* showed 35 DNA restriction fragment (Table-3) and allowed four unique species specific restriction profiles and *HaeIII* enzyme obtained 40 DNA restriction fragments (Table-3) of diazotrophic isolates indicating four unique species specific restriction pattern as obtained within the genus (Fig. 3c, d).

#### Pair-wise analysis

The characterization of diazotrophic native isolates and respective reference strains showed the genetic similarity on genetic base.

In *TaqI* enzyme, *Azotobacter* isolate ABA-1 and reference strain MTCC-446 showed 100% similarity with similarity coefficient of 1.00 (Table-4) i.e. appeared to have a closer relationship with each other than any other isolates examined. Similarly, reference strain MTCC-124 showed up to 50% similarity with native isolates ABA-1, ABA-14. Native diazotrophic isolate ABA-1 showed some distinct feature with other native isolates ABA-10 and ABA-2010 (Fig 4 a).

*Acetobacter* native isolate ACG-2 and reference strain MTCC-1226 showed 100% similarity with similarity coefficient 1.00 (Table-4) (Fig 4 a) while *Azospirillum* native isolate ASA-1 showed 50% similarity with reference strain MTCC-2306.

**Table 4.** The pair-wise correlation generated based on the restriction fragment for *TaqI*.

(a) <i>Azotobacter</i> cultures						
	ABA-1	ABA-10	ABA-14	ABA-2010	MTCC-446	MTCC-124
ABA-1	1.00					
ABA-10	0.14	1.00				
ABA-14	0.50	0.14	1.00			
ABA-2010	0.17	0.29	0.17	1.00		
MTCC-446	1.00	0.14	0.50	0.17	1.00	
MTCC-124	0.50	0.33	0.50	0.17	0.50	1.00

(b) <i>Acetobacter</i> cultures			(c) <i>Azospirillum</i> cultures		
	ACG-2	MTCC-1226		ASA-1	MTCC-2306
ACG-2	1.00		ASA-1	1.00	
MTCC-1226	1.00	1.00	MTCC-2306	0.27	1.00

**Table 5.** The pair-wise correlation generated based on the restriction fragment for *HinfI*

(a) <i>Azotobacter</i> cultures						
	ABA-1	ABA-10	ABA-14	ABA-2010	MTCC-446	MTCC-124
ABA-1	1.00					
ABA-10	0.40	1.00				
ABA-14	0.40	0.14	1.00			
ABA-2010	0.20	0.00	0.75	1.00		
MTCC-446	0.20	0.17	0.17	0.00	1.00	
MTCC-124	0.40	0.14	1.00	0.75	0.17	1.00

(b) <i>Acetobacter</i> cultures			(c) <i>Azospirillum</i> cultures		
	ACG-2	MTCC-1226		ASA-1	MTCC-2306
ACG-2	1.00		ASA-1	1.00	
MTCC-1226	1.00	1.00	MTCC-2306	0.50	1.00

**Table 6.** The pair-wise correlation generated based on the restriction fragment for *HaeIII*

(a) <i>Azotobacter</i> cultures						
	ABA-1	ABA-10	ABA-14	ABA-2010	MTCC-446	MTCC-124
ABA-1	1.00					
ABA-10	0.14	1.00				
ABA-14	0.50	0.14	1.00			
ABA-2010	0.17	0.29	0.17	1.00		
MTCC-446	1.00	0.14	0.50	0.17	1.00	
MTCC-124	0.50	0.33	0.50	0.17	0.50	1.00

(b) <i>Acetobacter</i> cultures			(c) <i>Azospirillum</i> cultures		
	ACG-2	MTCC-1226		ASA-1	MTCC-2306
ACG-2	1.00		ASA-1	1.00	
MTCC-1226	1.00	1.00	MTCC-2306	0.50	1.00

**Table 7.** Pooled pair-wise correlation

(a) <i>Azotobacter</i> cultures						
	ABA-1	ABA-10	ABA-14	ABA-2010	MTCC-446	MTCC-124
ABA-1	1.00					
ABA-10	0.20	1.00				
ABA-14	0.38	0.09	1.00			
ABA-2010	0.22	0.14	0.47	1.00		
MTCC-446	0.40	0.15	0.31	0.24	1.00	
MTCC-124	0.38	0.14	0.69	0.38	0.31	1.00

(b) <i>Acetobacter</i> cultures			(c) <i>Azospirillum</i> cultures		
	ACG-2	MTCC-1226		ASA-1	MTCC-2306
ACG-2	1.00		ASA-1	1.00	
MTCC-1226	1.00	1.00	MTCC-2306	0.27	1.00

In *Hinf*I enzyme, *Azotobacter* isolate ABA-14 and respective reference strain MTCC-124 showed 100% similarity with similarity coefficient 1.00 (Table-5) i.e. appeared to have a closer relationship of each isolates examined. Similarly, native isolates ABA-10 and reference strain MTCC-124 showed 75% similarity with ABA-2010. Native diazotrophic isolate ABA-1 showed some distinct feature with other native isolates ABA-10, ABA-41, ABA-2010 and reference strains MTCC-446 as well as MTCC124 (Fig 4b). Diazotrophic *Acetobacter* native isolate ACG-2 and respective reference strain MTCC-1226 showed 100% similarity (Table-7), while *Azospirillum* native isolate ASA-1 showed 50% similarity with reference strain MTCC-2306 (Fig. 4b).

In *Hae*III enzyme, *Azotobacter* isolate ABA-1 and respective reference strain MTCC-446 showed 100% similarity with similarity coefficient 1.00 (Table-6). Among 3 native isolates and 2 reference strains only ABA-14 and reference strain MTCC-124 showed 50% similarity with ABA-1 and other isolate showed some distinct feature (Fig 4c).

*Acetobacter* native isolate ACG-2 and respective reference strain MTCC-1226 showed 100% similarity at similarity coefficient 1.00 (Table-5) while *Azospirillum* native isolate ASA-1 showed 50% similarity with reference strain MTCC-2306. The products of *Taq*I and *Hae*III restriction endonucleases have been shown to discriminate between the *nifH* gene of several diazotrophic bacteria (Widmer *et al.*, 1999; Poly *et al.*, 2001) and shown a different RFLP profile for bacteria

belonging to the *Azospirillum*, *Burkholderia* and *Gluconacetobacter*.

*Bam*HI did not show the any restriction digestion pattern (Fig. 3b) on native diazotrophic isolates and reference strains also.

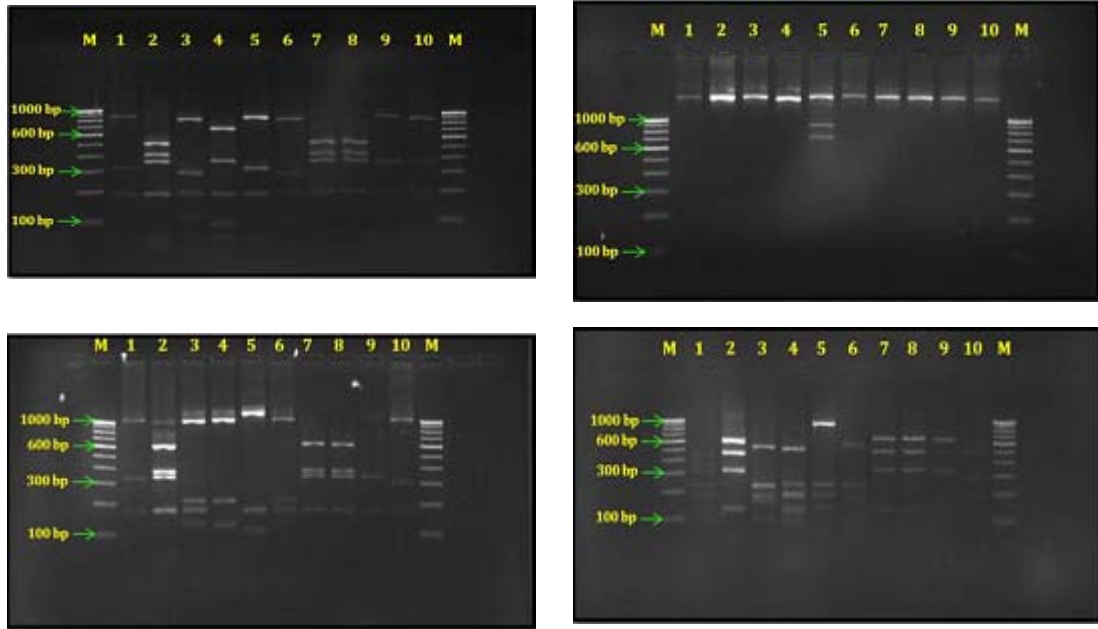
#### Pooled analysis

Pooled analysis of amplified ribosomal DNA restriction analysis of diazotrophic isolates and references strains is shown in dendrogram (Fig-4d).

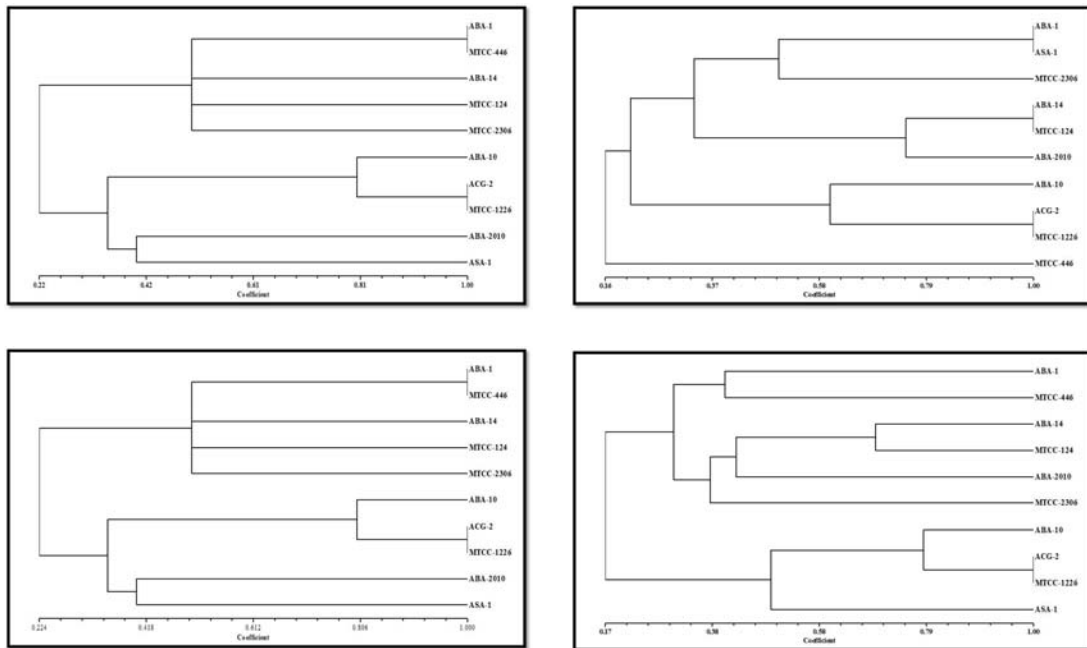
The diazotrophic isolate ACG -2 and reference strain MTCC-1226 (*A. diazotrophicus*) showed 100% similarity (coefficient of similarity 1.00) (Table-5) where as it was 69 % similarity of native isolate ABA 14 with reference strain MTCC-124 (*A. chroococcum*). Whereas, *Acetobacter* native isolate ACG-2 and reference strain MTCC-1226 (*A. diazotrophicus*) had 79% similarity with *Azotobacter* native isolate ABA-10 i.e. appeared to have a closer relationship with each other than other isolates examined. On the other side *Azospirillum* native isolate ASA-1 showed 50% similarity with *Acetobacter* native isolate ACG-2 and reference strain MTCC-1226.

Thus the data has shown that the diazotrophic native isolates of *Azotobacter* ABA-1, ABA-10, ABA-14 and ABA-2010 had some distinct features with each other and with related diazotrophic isolates of *Acetobacter* and *Azospirillum*. So, all native diazotrophic isolates of individual species having distinct features.

Over all, the ARDRA results indicates that similarity between diazotrophic isolate ACG -2 and reference strain MTCC-1226 (*A. diazotrophicus*)



**Fig. 3.** Restriction pattern of native diazotrophic bacterial isolates with (a) *TaqI*, (b) *BamHI*, (c) *HinfI* and (d) *HaeIII* enzyme



**Fig. 4.** Dendrogram of 10 diazotrophic isolates based on Amplified rDNA restriction analysis using unweight pair group method with arithmetic average (UPGMA) cluster analysis of (a) *TaqI*, (b) *HinfI*, (c) *HaeIII* enzyme and (d) Pooled



indicate 100% similarity. Whereas, 69 % similarity was found in native isolate ABA 14 with reference strain MTCC-124 (*A. chroococcum*). *Azotobacter* native isolate ABA-1, ABA-10, ABA-2010 also showed below 50% similarity with reference strain MTCC-446 & 124 (*A. chroococcum*) i.e. appeared to have some relationship with each other isolates examined. On the other side *Azospirillum* native isolate ASA-1 were distinct (27% similarity) feature with reference strain MTCC-2306 (*A. lipoferum*).

These results demonstrate the genetic diversity of microbial population assessed by measuring the heterogeneity of the DNA from the population in all the species of diazotrophic isolates based on 16S rDNA sequences directly amplified from an environmental sample. Separate universal primers are used to amplify bacterial and archeal ribosomal RNA genes using PCR. Digested fragments separated by electrophoresis generate characteristic profile data for estimation of diversity and the overall similarities between organisms. The greater the number of restriction bands, the greater is diversity. An algorithm helped in computerized alignment of sequences. Scoring was done as positive for a match and mismatch as zero and gaps as negative. Gaps were weighted more heavily than mismatch ensuring that not all the latter are eliminated by excessive insertion of gap. The construction of tree is based upon distance data using computer software by the unweighted pair group method with arithmetic averaging (UPGMA) using NTSYS software Phylogenetic analysis usually explains evolutionary history and relationship of microbes (dendrogram). Closely related microorganisms have similar sequences while distantly related microorganisms have sequences that are more dissimilar. Analysis of 16srDNA, known as amplified rDNA restriction analysis (ARDRA), can be used as a method for rapid comparison of r-DNAs obtained by PCR amplification by using universal primers.

### CONCLUSION

A comprehensive study was undertaken for preliminary characterization of native diazotrophic bacteria isolates viz. *Azotobacter*, *Acetobacter* and *Azospirillum* by ARDRA.

Pooled analysis of native diazotrophic isolates and reference strains by ARDRA showed

coefficient of similarity between diazotrophic isolate ACG -2 and reference strain MTCC-1226 (*A. diazotrophicus*) indicate 100% similarity where as 69 % similarity found in native isolate ABA 14 with reference strain MTCC-124 (*A. chroococcum*). On the other side *Azospirillum* native isolate ASA-1 showed 50% similarity with reference strain MTCC-2306 (*A. lipoferum*).

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