International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2017): 7.296

Diversity of *Azotobacter* Isolates from Different Rice Soils of Tamil Nadu

Sivapriya S.L[1], K. Kumar[2]

Abstract: ***Azotobacters are unique biofertilizers to maintain the N level in agricultural soil and synthesize the plant growth promoting hormones as indole acetic acid and gibberellins. Azotobacter EPS in the soil habitat play key roles in ecosystem functioning through controlling nutrient cycling reactions essential for maintaining soil fertility and also contributing to the genesis and maintenance of soil structure under conventional, biotic, and abiotic stressed soil environment. Each member of the bacterial population had varying responses to environmental factors and phenotypic variation in accordance with it genotype, so that required a grouping or classification to facilitate the study of bacterial diversity. The purposes of this study were to characterize Azotobacter species isolated from rice rhizospheres in different regions of Tamil Nadu and to determine the relationship between the species diversity of Azotobacter. The Azotobacterial isolates were characterized by utilizing a numerical taxonomy method based on phenotypic characters, including morphological, biochemical and physiological characters.The results indicated that AztRMD2 performed better than the standard strains. This study contributes to identify Azotobacter species isolated from rice rhizospheres in Tamil Nadu and to characterize EPS producing and cyst forming properties of Azotobacter spp. The results of this study may be of importance in allowing better utilization of Azotobacter biofertilizers.***

Keywords: *Azotobacter*, bacterial diversity, numerical taxonomy

1. Introduction

The Earth's population is expected to reach 10 billion by 2035 (DAE, 2006). It is clear that to maintain the current level of protein and calorific intake over the next 20 years, unprecedented increases in crop production are required. Furthermore, the climate changes and anthropogenic activities, such as urban development, road construction, industrial processes, mining and inadequate agricultural practices, are resulting in the eutrophication and pollution of soils and fresh water resources, soil degradation, loss of soil fertility, and desertification (McLauchlan, 2006; Spiertz, 2010; Gordon *et al*., 2010). So, augment in crop production will need to be achieved despite a significant deterioration of much prime agricultural lands and will require the utilization of large areas now considered marginal. Thus, BNF in agrosystems reduces the need for chemical nitrogen fertilizers and consequently reduces global warming and water contamination.

Nowadays, microbial polysaccharides are of great interest due to its wide industrial application and lack of structural complexity without extensive branching. The production of polysaccharides by microorganisms was first reported in 1880s. Applications of exopolysaccharides (EPS) are of continuing interest and have found a good commercial market in food and health care industry. The viscous exopolysaccharides produced by *Azotobacter* is well characterized (Gauri *et al*., 2009). *Azotobacter* is a free living, asymbiotic nitrogen fixer and mostly abundant in plant rhizosphere and phyllosphere region. The EPS of *Azotobacter* are copolyuronans ubiquitous nature of alginates (Elsayed *et al.,* 2013) commercially used in plant tissue culture to produce insoluble artificial seeds, immobilizing enzymes by entrapment, as food and wound dressing substances. Thus the industrial application of alginate is well understood (Hay *et al*., 2010). Aside from industrial application, *Azotobacter* alginate plays a key role in encystment, to provide protection against desiccation and predation by protozoa or phage attack (Hynes *et al*., 2008), or affect the penetration of antimicrobial agents and toxic metals (Aleem *et al*., 2003). This polysaccharide also protects nitrogenase against high oxygen concentration (Bhattacharyya and Jha, 2012) and also participates in interaction between plants and bacteria (Mandal *et al*., 2008).

The genus of *Azotobacter* is most important bacteria in sustainable agriculture system. A continuous biosynthesis and release of metabolites acting on soil structure and plant metabolism is a factor contributing to explain the increased soil fertility. The major issues in production of efficient biofertilizers have the characteristics of high rate of dinitrogen fixation, wide range of antagonistic activity towards phytopathogens, and the ability to produce EPS, siderophores, vitamins, and growth factors in agricultural prospective (Kravchenko *et al*., 2002). *Azotobacter* is the genus of great interest in agricultural application due to their free nitrogen fixing ability. Here, emphasis is given on the role of exopolysaccharide in sustainable agriculture system and also to the survival in its own environments.

EPS is more effective in binding or aggregating the soil. It also reduces the bulk density of Greenfield sandy loam and increased hydraulic conductivity in neutral soil. Moreover, the polysaccharide rich soil provides a cementing action to increase stability for soil aggregation (Flouri *et al.*, 1990). Soil aggregate stability increases biomass and decomposition of organic materials by PGPRs (Ahemad and Khan, 2012c; Tank and Saraf, 2010). This increased soil porosity and reduced bulk density allows retention and exchange of air and water. This pore space provides zones of weakness through which plant roots can grow easily.

This study will help to investigate the role of exopolysaccharide in sustainable agricultural system and also to the survival in its own environments. Hence, the present study have been made to isolate efficient EPS producing and cyst forming *Azotobacter* from different locations of Tamil Nadu, neither the diversity of Azotobacter species in paddy soils nor their biofertilizer potential has been thoroughly investigated.

2. Materials and Methods

All the laboratory experiments were conducted at the Department of Agricultural Microbiology, Tamil Nadu Agricultural University (TNAU), Coimbatore. All the enzymes and primers used in molecular studies were obtained from M/s. Bangalore Genei(P) Ltd., Bangalore and Sigma Aldrich, USA. The Analytical Reagent Grade (AR) chemicals were obtained from M/s. Himedia, M/s. Qualigens, M/s. Merck, M/s. BDH and M/s. Sigma and were used for media preparation and biochemical studies.The standard strain of *Azotobacter chroococcum* (Ac1) maintained at the Biofertilizer Production and Quality Control Laboratory, TNAU and the standard strain of *Azotobacter venilandii* (MTCC2460) was obtained from Microbial Type Culture Collection and Gene Bank, India were used in the study.

2.1. Collection of soil sample

The rhizosphere soil samples were collected from different rice growing places such as Paramakudi, Madurai, Ramnad and different region of Kanyakumari and Nilgiri districts. Rhizosphere soil samples were collected carefully by uprooting the root system and placed in a cool box for transport and stored at 4° C.

2.2. Physico- chemical analysis of soil

Table 1: Methods employed for Physico- chemical analysis of soil

Particulars	Method	Reference
pH(Soil: Water = 1:2)	Using glass electrode in the ELICO pH meter	Jackson (1973)
Electrical conductivity (Soil: Water = 1:2)	Using ELICO conductivity bridge	Jackson (1973)
Texture	Robinson's international pipette method	Piper(1966)
Available N	Alkaline permanganate method	Subbiah and Asija(1956)
Available P	Using 0.5 M $NaHCO_3$ of pH 8.5 using colourimeter	Olsen *et al*.,(1954)
Available K	Flame photometric method using neutral normal ammonium acetate extract	Stanford and English(1949)

2.3. Isolation and enumeration of *Azotobacter* from rhizosphere soil

Azotobacter was enumerated and isolated by following pour plate method (Allen, 1953). The Waksman No 77 medium was used for enumeration and isolation of *Azotobacter*. One gram of soil from each sample was aseptically weighed, transferred to 100ml sterile water blank and shaken (120 rpm) form 0 min to get 10^{-2} dilution. After thorough shaking, one ml of diluents from 10^{-2} was transferred to 9 ml water blank to get 10^{-3} dilution. Likewise the sample was diluted serially with 9 ml water blanks until the appropriate dilution was obtained. Aliquots (1ml) from the serially diluted samples (10^{-3} to 10^{-6}) were added to N-free media in Petri plates and kept in an incubator at 30°C for isolation and enumeration.

2.4. Purification and designation of the isolates

Colonies with similar colony characters were grouped according to their morphological characteristics. Single colonies were picked from the Petri dishes and sub-cultured several times to obtain pure cultures. Stock cultures were made in nutrient broth containing 50% (w/v) glycerol and stored at -80°C.A total of 30 cultures, isolated from the rhizosphere soil from different locations in Tamil Nadu, were purified and used for confirmation of nitrogen fixing ability.

2.5 Characterization of the isolates

2.5.1 Morphological tests
The following morphological tests were carried out for the 30 isolates for identification of the genus.

2.5.2 Colony characters
The colony characters *viz.*, colour, shape, size and colony character were observed on Waksmann No 77 medium (Gerhardt *et al*., 1981).

2.5.3 Gram Stainning
Gram staining of the isolates was carried out as per Huckers modified method (Rengaswami, 1975).

Thin smears of the culture were made on separate glass slides, air dried, heat fixed and the smear was stained with crystal violet for 1 min, followed by a wash with distilled water for few seconds. It was then treated with lugols iodine solution for 30 seconds and decolorized with 95% ethyl alcohol, followed wash with distilled water and dried. The smear was then counterstained with safranin for 30 seconds, again washed with distilled water blot dried, air dried and the cellular morphology was observed under microscope.

2.5.4. Motility test (Skerman, 1969)
Motility of the isolates was tested using motility medium. The culture was stabbed at the centre and incubated at 28°C and 24 hours. Motility strains were identified by diffused growth in to medium away from stab line.

2.5.5. Pigmentation of the isolates
The bacterial isolates were observed for the characteristic pigmentation of the colonies.

2.5.6. Biochemical characterization of the isolates
The following biochemical tests were carried out.

2.5.7. Catalase test (Smibert and Krieg, 1981)
Cultures are grown on nutrient agar slants 24 to 48h and flooded with 0.5 ml of 3% hydrogen peroxide. Rapid effervescence showed positive result for catalase activity.

2.5.8. Oxidase test (Collins and Lyne, 1970)
Small pieces of filter paper were soaked in 1% aqueous tetra methyl-p-phenylene diamine and placed in a Petri dish. Fresh young cultures to be tested were scraped with a glass rod and rubbed on the moistened filter paper. Development of a deep violet colour after 10 sec indicated positive oxidase test, whereas development of a light violet or no colour indicated negative oxidase test.

2.5.9. Indole formation test (Gillus, 1956)
The indole test was performed by inoculating the bacterial isolates into Tryptone broth. The indole production during the growth was detected by adding Kovac's reagent (P-dimethyl amino benzaldehyde), which produced cherry red colour for positive reaction.

2.5.10. Starch hydrolysis test (Seeley and Van Demark, 1981)
The isolates were streaked on nutrient agar plates containing 2 per cent insoluble starch and incubated at room temperature for 48 h. Hydrolysis of starch was tested by flooding with Gram's iodine solution and the plates were observed for the presence a clear zones surrounding the colonies, which is a positive indicator for starch hydrolysis.

2.5.11.Gelation liquefaction (McDade and Weaver, 1959**)**
The proteolytic activity is determined by growing the isolates on GYP agar medium supplied with 0.4% gelatin and sterilized. To the sterilized culture media, separately sterilized gelatin (8g of in 100ml distilled water) was added along the. After incubation, the grown culture was flooded with saturated aqueous ammonium sulphate. The clear zone around the colony indicates the hydrolysis of gelatin in media and the unhydrolysed gelatin is precipitated by ammonium sulphate.

2.6. Diversity study of *Azotobacter* isolates

2.6. 1. Isolation of genomic DNA
The genomic DNA from the seventeen isolates was isolated using the standard protocol of hexadecyl-trimethyl ammonium bromide (CTAB) method given by Melody (1997) with slight modifications. Actively grown culture of 25 ml quantity was taken in a centrifuge tube and centrifuged at 6,000 rpm for 5 min at 4^0 C. The supernatant was removed, the pellet was suspended in 1 ml TE buffer, added with 0.5 ml of 1-butanol, vortexed well to mix with the cells (to remove extracellular materials). Centrifuged at 5000 rpm for 5 min at 4^0C, the supernatant was discarded and the pellet was resuspended in 2 ml of TE buffer and centrifuged again to remove all traces of butanol. Again the pellet was resuspended in 1 ml TE buffer added with 100 µl lysozyme (10 mg ml^{-1} freshly prepared) and incubated at room temperature for 5 min. After incubation, 100 µl of 10 per cent SDS and 25 µL of 100 µg ml^{-1} proteinase K were added, mixed well and incubated at 37 ^{0} C for 1 h. To this 200 µl of 5 M NaCl was added and mixed well. CTAB solution in 150 µl quantity was added, mixed well and incubated at 65^0 C for 10 min. The mixture was extracted with 1 ml of phenol: chloroform mixture, mixed well and centrifuged at 6000 rpm for 15 min at 4^0C. The aqueous layer was transferred carefully to a 2.0 ml microfuge tube and DNA was precipitated by adding 0.6 volume of ice cold isopropanol, incubated 1 h to overnight at -20^0C. The DNA was pelletized by centrifugation at 12000 rpm for 15 min at 4^0C. The pellet was washed with 70 percent ethanol, dried under vacuum for 10 min and resuspended in 50 µl of TE buffer. One µl DNAse free RNAse (10mg per ml) was also added by swirling and incubated at 37^0C for 30 min. The DNA was stored at -20^0C for further use (Annexure II).

2.6.2. Agarose gel electrophoresis (Sambrook *et al.*, 1989)
Agarose gel electrophoresis was performed based on the method given by Sambrook *et al*. (1989) to check the quality of DNA and also to separate the products amplified through polymerase chain reaction. 1X TAE (Annexure II) tank buffer in 500 ml quantity was prepared to fill the electrophoresis tank and to prepare the gel. In a separate conical flask, agarose (0.8 per cent for genomic DNA and 1.5 per cent for PCR product) was added to 1X TAE buffer, boiled till the agarose dissolved completely and cooled to lukewarm temperature. Ethidium bromide was added at the rate of 5 µl 100 ml^{-1} to agarose solution and was allowed to mix completely. It was then poured into the gel mould and the comb was placed properly, allowed to solidify for half an hour at room temperature. After solidification the comb was removed carefully. The caste gel was placed in the electrophoresis tank containing 1X TAE buffer with the well near the cathode and submerged to a depth of 1 cm. Fifteen µl of the PCR product was mixed with 3µl of 6X tracking dye (Annexure II) and mixed well by pipetting in and out for 3 times. The mixture was loaded into the wells with the help of the micropipette. Two µl of 1 kb DNA ladder (Fermentas, USA) was loaded in one of the wells as a standard marker. The cathode and anode were connected to power pack using power cord and the gel was run at a constant voltage of 60 volts. The negatively charged DNA molecules move towards the anode and get separated according to their molecular weight. The power was turned off when the marker reached the anode end and the gel was viewed in an UV transilluminator and the banding pattern was analyzed.

2.6.3. ARDRA profiling of Azotobacter isolates
Nearly-full length of 16S rRNA gene was amplified from the genomic DNA from each isolate using FD1 (5'AGA GTT TGA TCC TGG CTC AG 3') and RP2 (5'ACG GCT ACC TTG TTA CCA CTT 3') primers (Weisburg *et al.*, 1991). A total of 20 µL of reaction volume contains 50 ng of genomic DNA, 0.2 mM of each dNTP, 1 µM of each primer, 2.5 mM of $MgCl_2$ and 1 U of Taq DNA polymerase (all from Bangalore Genei, India) and the buffer supplied with the enzyme. PCR amplification was performed in a thermocycler (Eppendorf Master Cycler, Germany) using conditions as follows: initial denaturation at 95ºC for 10 min; 35 cycles consisting of 94ºC for 1 min (denaturation); 55ºC for 1 min (annealing); 72ºC for 1 min (primer extension) and final extension at 72ºC for 10 min. Approximately 1 µg of PCR-amplified 16S rRNA gene fragments were restricted with endonuclease HaeIII (Fermentas, USA) at 37 ºC for 3h and resolved by electrophoresis in 2% metaphor agarose gels. Banding patterns were visualized by ethidium bromide staining and documented in Alpha Imager TM1200 documentation and analysis system.

2.6.4. ERIC-PCR fingerprinting Analysis of Azotobacter isolates
ERIC-PCR was done in the seventeen Azotobacter isolates with the specific primers ERIC1R (5'-ATG TAA GCT CCC TGG GGA TTC AC- 3') and ERIC2L (5'AAG TAA GTG ACT GGG GTG AGC G- 3'). A total of 20 µl of reaction volume contains 50ng of genomic DNA, 0.2 mM of each dNTP, 1µM of each primer, 2.5 mM of $MgCl_2$ and iU of Taq DNA polymerase (all from Bangalore Genei, India) and the buffer supplied with the enzyme. PCR amplification was

performed in a thermocycler (Eppendorf Master Cycler, Germany) using conditions as follows: initial denaturation at 95 ºC for 10 min; 35 cycles consisting of 94ºC for 1 min (denaturation); 53ºC for 1 min (annealing); 72ºC for 8 min (primer extension) and final extension at 72ºC for 15 min.

2.6.5. BOX-PCR fingerprinting Analysis of Azotobacter isolates

BOX-PCR was done in the seventeen Azotobacter isolates with the primer BOX (BOX1R 5'-CTA CGC CAA GGC GAC GCT GAG- 3'), A total of 20 µl of reaction volume contains 50ng of genomic DNA, 0.2mM of each dNTP, 1 µM of each primer, 2.5 mM of $MgCl_2$ and iU of Taq DNA polymerase (all from Bangalore Genei, India) and the buffer supplied with the enzyme. PCR amplification was performed in a thermocycler (Eppendorf Master Cycler, Germany) using conditions as follows: initial denaturation at 95 ºC for 10 min; 35 cycles consisting of 94ºC for 1 min (denaturation); 53ºC for 1 min (annealing); 72ºC for 8 min (primer extension) and final extension at 72ºC for 15 min.

2.6.6. Dendrogram analysis of Azotobacter isolates

In order to determine the similarity between the isolates within the soil sample, a binary matrix was established recording the presence or absence of band in ARDRA profile, ERIC and BOX PCR. UPGMA algorithm was used for hierarchical cluster analysis. Pair wise comparisons were calculated using Jaccard's coefficient and dendrogram was built using the UPGMA method using NTSYS-PC2 package (Numerical taxonomy analysis program package, External software, USA).

3. Experimental Results

Nitrogen (N) is a constituent of various cellular components, such as amino acids, proteins, enzymes, nucleic acids and chlorophyll. Numerous fundamental biochemical reactions involve the presence of N, which is the fourth most-consumed nutrient of cultivated plants. A wide diversity of nitrogen fixing bacterial species has the capacity to colonize the rhizosphere and to interact with plants.Plant associated nitrogen-fixing bacteria have been considered as one of the possible alternatives for inorganic nitrogen fertilizer for promoting plant growth and yield. The varieties of nitrogen fixing bacteria have been isolated from the rhizosphere region of various crop plants. In the present study, attempts were made to isolate the *Azotobacter* from the rhizosphere soil samples of rice. The isolates were purified and characterized. Selected isolates were screened for polysaccharide production, cyst formation, ability to form soil aggregates and also evaluated for their performance in rice by pot culture experiment. The major findings of these experiments are presented below.

3.1 Physico-chemical properties of rhizosphere soil samples of rice grown in different location of Tamil Nadu

Various physico-chemical characters *viz.*, pH, EC, texture, available nitrogen, phosphorus and potassium were estimated. The rhizosphere soils of rice from different locations were neutral to strong alkaline with pH ranging between 7.0 and 9.5 and EC ranging between 0.12 and 0.90 dSm^{-1}. The available nitrogen and phosphorus was medium and the potassium was low to medium in all the locations.

3.2 Isolation and purification of *Azotobacter* isolates from different locations of Tamil Nadu

Seventeen *Azotobacter* strains were isolated from different types of soil samples collected from various parts of Tamil Nadu such as Ramnad, Paramakudi, Madurai, Kanyakumari and Nilgiris districts. The *Azotobacter* isolates were purified by repeated streaking on Waksman No 77 agar and selected from single pure colonies (Plate 1). These isolates were designated based on the location from which they were isolated. Purified isolates were maintained in Whatsman No 77 agar slants at 4ºC for further studies.

3.3 Pigment production

Out of seventeen isolates, ten isolates were able to produce brown to black coloured pigments and 7 other isolates found to be lacking this capability. Based on the pigment formation, ten isolates were selected for further study.

3.4 Morphological characterization of *Azotobacter* isolates

3.4.1. Characterization of *Azotobacter* isolates based on cell morphology

Based on the pigment formation, ten *Azotobacter* isolates were characterized by their cell morphological characters *viz.*, vegetative cell size, shape and motility. The results are presented in Table 3 and Plate 2. *Azotobacter* isolates AztPMK2 appeared as medium sized and rod shaped cells. AztPu1, AztPa1, AztMDU1, AztKa , AztMDU2,and AztMDUT1 isolates were small sized and rod shaped to ovoid shaped cells. AztPMK1, AztRMD1 and *Azt*RMD2 isolates were large sized and rod shaped cells. When grown in Waksman No 77 broth, all the isolates formed pellicle. These morphological characters of isolates were similar the standard strain *Azotobacter venilandii* (AztMTCC 2460) and *Azotobacter chroococcum* (AztTNAU). All the *Azotobacter* isolates were gram negative. The cell shape of the isolates varied from rods to ovoid shapes, reflecting the pleomorphic nature of *Azotobacter*.

3.4.2. Characterization of *Azotobacter* isolates based on colony morphology

Ten *Azotobacter* isolates were identified based on their colony morphology also(Table 1). The colonies of most of the *Azotobacter* isolates were irregular in shape, large to moderate in size and brown colored pigment producing mucoid colonies.

Table 1: Colony characters of *Azotobacter* isolates

Azotobacer Isolates	Morphological characters							
	Shape	Size	Pigmentation	Margin	Texture	Elevation	Appearance	Optical property
AztPKM1	Irregular	Large	Intense Brown	Curled	Rough	Convex	mucoid colonies	Opaque
AztPu1	Irregular	Moderate	light Brown	Curled	Rough	Convex	mucoid colonies	Opaque
AztPKM 2	Irregular	Large	Medium Brown	Entire	Rough	Umbonate	mucoid colonies	Opaque
AztPa1	Circular	Moderate	light Brown	Curled	Smooth	Convex	mucoid colonies	Opaque
AztMDU1	Irregular	Large	light Brown	Entire	Rough	Umbonate	mucoid colonies	Opaque
AztKa	Circular	Moderate	light Brown	Curled	Smooth	Umbonate	mucoid colonies	Opaque
AztMDU2	Irregular	Moderate	light Brown	Undulated	Rough	Umbonate	mucoid colonies	Opaque
AztMDU1	Irregular	Moderate	light Brown	Curled	Smooth	Umbonate	mucoid colonies	Opaque
AztRMD1	Irregular	Large	Intense Brown	Curled	Smooth	Convex	mucoid colonies	Opaque
AztRMD 2	Irregular	Large	Intense Brown	Undulated	Rough	Convex	mucoid colonies	Opaque
AztTNAU*	Irregular	Moderate	Medium Brown	Undulated	Rough	Convex	mucoid colonies	Opaque
AztMTCC 2460*	Irregular	Moderate	Yellowish green	Entire	Smooth	Convex	mucoid colonies	Opaque

3.5. Biochemical characterization of *Azotobacter* isolates

For further authentication of the isolates, the following biochemical tests were carried out and the results are presented in Table 2. All the biochemical test such as catalase, oxidase, indole formation, nitrate reductase and gelatin liquefaction were positive for all the selected *Azotobacter* isolates. Among the 10 isolates, AztPMK 1, AztPMK 2, *Azt*RMD2 and the two standard strains AztMTCC 2460 and AztTNAU were highly positive for the above biochemical tests.

Table 2: Biochemical characteristics of selected *Azotobacter* isolates

Azotobacter Isolates	Biochemical tests					
	Catalase test	Oxidase test	Indole formation	Starch hydrolysis	Gelatin liquefaction	Nitrate reductase
AztPKM1	+++	+++	+++	+++	+++	+++
AztPu1	+	+	++	++	+	+
AztPKM 2	+++	+++	+++	+++	+++	+++
AztPa1	+	+	+	+	+	+
AztMDU1	+++	++	+++	+++	++	++
AztKa	+	+	+	+	+	+
AztMDU2	+	+	+	+	+	+
AztMDUT1	+	+	+	+	+	+
AztRMD1	+++	+++	+++	+++	+++	+++
AztRMD 2	+++	++	+++	+++	++	++
AztTNAU*	+++	+++	+++	+++	+++	+++
AztMTCC 2460*	++	++	+++	+++	++	++

3.6. Carbon-substrate utilization by *Azotobacter* isolates from rhizosphere soil of rice

Azotobacter isolates are capable of growing on various carbon compounds such as D -glucose, fructose, mannitol, sucrose, maltose,and galactose. The results on the utilization of different carbon substrates by the isolates are presented in Table 3. Glucose, fructose and mannitol were found to be the most preferred carbon source for *Azotobacter* isolates, but lactose was not utilized by *Azotobacter*.

Table 3: Carbon utilization by *Azotobacter* isolated from different locations in TamilNadu

Azotobacter Isolates	Glucose	Sucrose	Lactose	Fructose	Galatose	Manitol
AztPKM1	+	+	-	+	+	+
AztPu1	+	+	-	+	+	+
AztPKM 2	+	+	-	+	+	+
AztPa1	+	+	-	+	+	+
AztMDU1	+	+	-	+	+	+
AztKa	+	+	-	+	+	+
AztMDU2	+	+	-	+	+	+
AztMDUT1	+	+	-	+	+	+
AztRMD1	+	+	-	+	+	+
AztRMD 2	+	+	-	+	+	+
AztTNAU*	+	+	-	+	+	+
AztMTCC 2460*	+	+	-	+	+	+

3.7. Diversity of *Azotobacter* isolates from different rice soils of Tamil Nadu

A total of seventeen *Azotobacter* isolates showing typical growth pattern in Waksman No 77 medium. All the *Azotobacter* isolates with standard strain (*Azotobacter chroococcum* and *Azotobacter venilandii*) were extracted for genomic DNA by CTAB method after culturing overnight in Tryptic soy broth at 30 ˚C. The DNA was intact and the concentration was nearly uniform in all the isolates. The

genomic DNA was used as template for the amplification of 16S rRNA gene using universal primers, FD1 and RP2 primers. PCR fragments of expected size (1500 bp) were observed for all isolates tested, including positive control (*Azotobacter chroococcum* and *Azotobacter venilandii*). As expected, the amplification was absent in the negative control reaction (water).

The ARDRA profiling of seventeen selected *Azotobacter* isolates was developed approximately, 1 µg of PCR-amplified 16S rRNA gene fragments were restricted with endonuclease *Hae*III and the profiles were compared in the form of dendrograms developed by Jaccord's similarity index (Fig. 4). The dendrogram showed that the diversity of *Azotobacter* isolates in the rice ecosystem. It was recorded 11 groups of *Azotobacter* isolates (while using 0.6 Jaccord's similarity index to discriminate as a cluster). Out of seventeen Azotobacter isolates AztPMk1 and *A.Venilandii* was presented in group I, AztPMk2 in group II, A.chroococcum in group III, AztPMk3 and AztPu1 in group IV, AztMDU2 in group V, AztMDU1, AztRMD2, AztPu2 and AztPo1 in groupVI, AztMDU3 in group VII, AztRMD1 in group VIII, AztPa2 in group IX, AztPu3 in group X and finally AztMDUT1 and AztMDUT2 in group XI (fig 1).

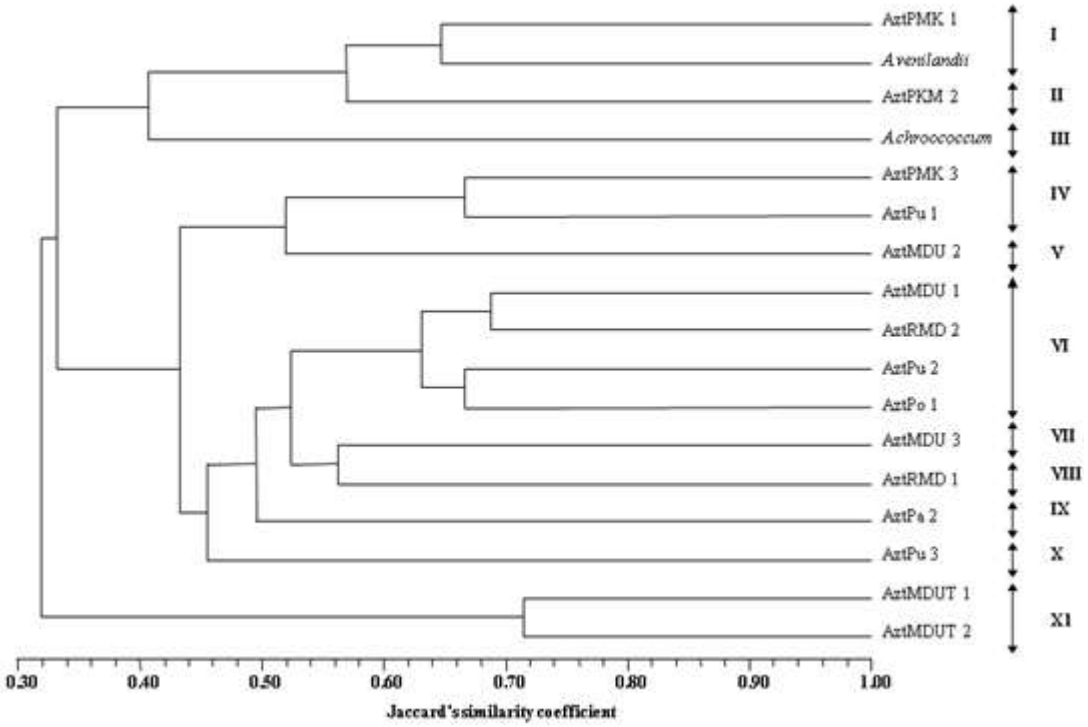

Figure 1: Dendrogram showing clustering of 15 Azotobacter isolates from rhizosphere soil from different rice growing regions in Tamil Nadu. The binary data showing based on the ARDRA, BOX, ERIC banding patterns were used for clustering the isolates by Jaccard's similarity index. The coefficient >0.60 was used to group the isolates into different groups

4. Discussion

Maintenance of soil health is the fore most important aspects for increasing crop productivity with sustainability. Improper and indiscriminate use of inorganic fertilizers without organic amendments continually over a period of time definitely affecting the soil health and crop productivity. This resulted that the productivity reached plateau. The reasons for this catastrophy in India are many fold, like majority farmers are marginal, shrinking of cultivable land in over a period of time force the farmers to go for intensive agriculture without caring for green/green leaf manuring. Cost estimation coupled with short fall in supply chain of inorganic fertilizers etc. It is evident that exploitation of soil without caring the soil health would definitely hamper the soil quality (Sanders, 1992). Proper use of balanced fertilization would improve the soil physio-chemical properties (Mandal *et al.*, 2007), microbial (Kuzyako, 2002) and enzymes activity (Mandal *et al.*, 2007). Indian soils are low in organic matter content, ranged from 0.21 to 1.46 per cent. It is difficult to increase the organic matter over night. Only long-term amendment of soil with adequate quantity of organic manuring will be the key to address this acute problem (Meng *et al.*, 2005). Efforts are on to address this by creating awareness about the use of organic manuring for building up and restoring the soil fertility. For these, lot of scientific validation about are required to find out (change in soil profile) (Both physio-chemical and microbiological) with and without organic amendments. Monitoring the changes in soil physio-chemical properties, organic matter build-up, biological processes, enzymes activity and microbial diversities in soil affected by these nutrient management systems is very much essential to keep the soil sustainability. With this background, the present investigation was carried out to assess the changes in soil physio-chemical properties with special reference to microbial community diversity and biological processes such as soil enzyme, nitrification potential, substrate induced respiration etc. due to long term addition of organic manures and synthetic fertilizers. The culture independent metagenomic approach targeting 16SrRNA gene for the eubacterial community diversity along with culturable- and functional bacterial communities were accessed in soil. Apart from these the abundance of eubacterial community and some nitrogen cycling gene

including *nifH* (Nitrogen fixation) and *amoA* (Nitrification) were also accessed in the soil by quantitative PCR. The results obtained from these experiments are discussed in this chapter.

4.1. Improving soil physio-chemical properties through long-term organic manure amendments

4.1.1. Inference on physio-chemical and nutrient availability

Physio-chemical properties of the soil imply the soil health and fertility which inturn reflect the crop growth and yield. The soil physio-chemical properties are highly influenced by crop management, fertilizer application, tillage practices and organic manure content (Pernes-Debuyser and Tessier, 2004). Under intensive cultivation, deterioration of soil physio-chemical properties is very often associated with decrease in organic matter (Anderson *et al* 1990). Hence the organic matter content is most frequently used as an indicator to discuss the physio-chemical properties(Chenu*etal.*,2000). In general, it is widely accepted that continuous application of both organic manures and inorganic fertilizers had significant effect on soil pH, available macro (NPK) nutrients and micronutrients (Fe, Zn, Mo, Mn). Among the two different nutrient management systems *i.e.*, organic management of nutrition and conventional inorganic fertilization, the former enhances the nutrient availability for a long term, while the latter one have a short term effect (Stark *et al.*, 2007). The results of the present investigation suggested that continuous application of organic manures significantly increased the available nutrients in soil than chemical fertilizer amended- and unfertilized control soils. However the nutrient status of inorganic fertilizer applied and unfertilized soils are on par. Continuous application of chemical fertilizers at recommended dosage did not cause deterioration of soil physio-chemical properties. The result of these were consistent for both the assessment years and in accordance with earlier finding (Masto *et al*., 2006).

4.2 Changes in soil microbiological property

The soil microbial biomass (MBC), which represents about 1 - 5% of total soil organic carbon, can provide an effective early warning of the improvement or deterioration of soil quality as a result of different management practices (Powlson and Brooks, 1987). In the present study, MBC was highest in the farmyard manure followed by inorganic fertilizer treatment. An increased MBC content after farmyard application was also reported by Marschner *et al*. (2003). Hao *et al*. (2008) observed in three subtropical paddy soils that the microbial biomass was considerably greater in soils receiving farmyard manure along with NPK fertilizer than in plots receiving merely NPK fertilizer. In addition to general quantitative difference between OM and IC, the microbial biomass - C levels were greatly enhanced in OM than IC and control. Straw plus inorganic fertilizer also increased the MBC and MBN. Continuous application of organic amendments enhance the soil microbiota, which play an important role for soil characteristics since many of them are involved in nutrient cycling, transformation processes and soil aggregate formation as well as plant growth promotion (Fernandes *et al*., 1997; Bastida *et al*., 2008). Liu *et al*. (2010) found lowest ratios in the N or control soils, and the highest in the NP+FKM treatment. Similarly, MBC: SOC ratio was highest in soil receiving continuous (7 years) poultry manure and lowest in soils receiving chemical fertilizers (Kaur *et al*., 2005). Moreover, green manuring is considered a good agricultural practice because of its positive effect on soil fertility, quality and biodiversity (Stark *et al*., 2007). In the short-term, several studies supported the evidence of an increase of microbial biomass and activity under organic management, leading to high nutrients availability for plants (Zaman *et al*., 1999; Tu *et al*., 2003; Wang *et al*., 2004; Marinari *et al*., 2006). Tu *et al*. (2003) showed a clearcut relationship between soil microbial biomass and activity with increased high net N mineralization rates, which resulted in larger N availability. Some experiments have compared organic and conventional agricultural systems through examining their effects on soil microbial biomass, microbial activity and substrate utilization and documented a higher microbial biomass in plots with organic amendments (Hu *et al*., 1997; Gelsomino *et al*., 2004). Sustained increases in microbial biomass resulting from high organic matter inputs have also been observed in organic and low-input systems (Bossio *et al*., 1998).

4.3. Microbial diversity as influenced by long-term nutrient managements

4.3.1. Diversity of *Azotobacter*

In the present investigation, genetic diversity of free-living diazotroph, *Azotobacter* as influenced by long-term addition of organic manures and chemical fertilizers in semi-arid alfisol soil was assessed. The reason to study this organism is that application of *Azotobacter* as a potential bioinoculant has been reported to improve crop yields of both annual and perennial crops such as cotton, wheat, oilseeds and cereals (Lakshminaryana 1993; Mrkovacki *et al*. 2001; Narula *et al*. 2005 a and b). However, its establishment in the rhizosphere and survival in the soil are known to be affected by climatic conditions, plant species and organic matter content of the soil (Dart and Subba Rao 1981; Rajakumar and Lakshmanan 1995). Soil properties and conditions like available nitrogen, soil moisture and soil temperature also affect the various physiological properties of *Azotobacter*.

The common molecular diversity method for culturable bacterial diversity, ARDRA has been employed to assess the *Azotobacter* diversity in soil. ARDRA is a widely used technique for microbial diversity analysis and it has the potential to discriminate the bacteria at the species level. This method involves amplification of the 16S ribosomal DNA region followed by digestion with one or more selected restriction enzymes (Heyndrickx *et al*. 1996). In the past, this technique has been already used successfully to discriminate the diversity of *Methylobacterium* in phyllosphere of plant species (Balachandar *et al*., 2008; Raja *et al*., 2008). In this study, the ARDRA profiling of 20 selected *Azotobacter* isolates from each soil was developed using *HaeIII* enzyme and the profiles were compared in the form of dendrograms developed by Joccord's similarity index. The dendrograms clearly showed not much difference in the diversity of *Azotobacter* in the soil affected by even 100 years of continuous application of either organic manures or chemical fertilizers. The OM soil and control soils recorded 15 clustering of *Azotobacter* isolates (while

using 0.9 Jaccard's similarity index to discriminate as a cluster) each, whereas the chemical fertilizers amended soils had 13 clusters. Bhatia *et al*. (2009) assessed the *nifH* based diversity analysis of *Azotobacter* by Restriction Fragment Length Polymorphism in different cotton fields of India and revealed that they have large commonality in their population across the country. Similar investigation made on *Azotobacter* diversity in soil treated with long-term addition of waste water containing heavy metals resulted no damage to their diversity, rather the strains responded to long-term application of industrial waste water by an increase in resistance and maintained and survived in contaminated environment (Aleem *et al*., 2003). In this study also, *Azotobacter* present in the soil withstand and maintain their diversity potential even after continuous addition of chemicals including nitrogenous fertilizers. Similar diversity analysis of soil actinobacteria, whose activity similar to *Azotobacter* influenced by anthropogenic activities, affected by long-term organic- and inorganic soil amendments was assessed using 16S rRNA fingerprinting by DGGE (Piao *et al*., 2008) and it was shown that long-term organic and inorganic soil amendments did not significantly alter the phylogenetic diversity of the actinobacterial communities but did significantly change the community structure.

5. Conclusion

The important observation made in the present investigation is that continuous application of chemical fertilizers at proper dosage to the crop in semi-arid alfisol soil did not cause any deleterious effect to *Azotobacter* diversity. The present results also emphasis the use of optimum dose of chemical fertilizers to enhance the crop growth without affecting the soil microbial diversity and use of diversified organic manures to increase the biological factors for long-term sustainability and productivity of semi-arid alfisol soils.

References

[1] Ahemad, M. and M.S. Khan. 2012c. Evaluation of plant growth promoting activities of rhizobacterium *Pseudomonas putida* under herbicide-stress. **Ann. Microbiol., 62:** 1531–1540.

[2] Ahemad, M. and M.S.Khan.2012a. Effect of fungicides on plant growth promoting activities of phosphate solubilizing *Pseudomonas putida* isolated from mustard (*Brassica campestris*) rhizosphere. **Chemosphere., 86:** 945–950.

[3] Ahemad, M., and M.S.Khan. 2010a. Influence of selective herbicides on plant growth promoting traits of phosphate solubilizing *Enterobacter asburiae* strain PS2. **Res. J. Microbiol., 5:** 849–857.

[4] Ahemad, M., and M.S.Khan.2010b. Plant growth promoting activities of phosphate-solubilizing *Enterobacter asburiae* as influenced by fungicides. **Eurasia. J. Biosci., 4:** 88–95.

[5] Ahemad, M., and M.S.Khan.2012e. Alleviation of fungicide-induced phytotoxicity in greengram [*Vigna radiata* (L.) Wilczek] using fungicide-tolerant and plant growth promoting *Pseudomonas* strain. **Saudi J. Biol. Sci., 19:** 451–459.

[6] Aleem, A., J. Isar and A. Malik.2003. Impact of long-term application of industrial wastewater on the emergence of resistance traits in *Azotobacter chroococcum* isolated from rhizospheric soil. **Bioresour. Technol., 86:**7–13.

[7] Alavi, P., M.R. Starcher, C. Zachow, H. Müller and G. Berg. 2013. **Root-microbe systems: the effect and mode of interaction of stress protecting agent (SPA) Stenotrophomonas rhizophila DSM14405^{T}.** *Front. Plant Sci.*, **4:**141.

[8] Bhattacharyya, P.N. and D.K. Jha. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. **World J. Microbiol. Biotechnol., 28:** 1327–1350.

[9] Chandler, D., G.Davidson, W.P. Grant, J. Greaves and G.M. Tatchell. 2008. Microbial biopesticides for integrated crop management: an assessment of environmental and regulatory sustainability. **Trends Food Sci. Tech., 19:** 275–283.

[10] Elsayed, N.S., M. Aboulwafa, K. Aboshanab and N. Hassouna. 2013. PHB production in *Azomonas*, *Acinteobacter* and *Bacillus* species: isolation, screening and identification. **Arch. Clin. Microbiol.,** In press**.**

[11] Fatnassi, I.C, S.H.Jebara and M. Jebara. 2011. Selection of symbiotically efficient and high salt-tolerant rhizobia strains by gamma irradiation. **Ann Microbiol., 61:** 291-297.

[12] Freitas, F., V.D. Alves and M.A.M. Reis. 2011. Advances in bacterial exopolysaccharides: from production to biotechnological applications. **Trends Biotechnol., 29:**388–398.

[13] Gauri, S.S., S. Archanaa, K.C. Mondal, B.R. Pati, S.M. Mandal and S. Dey. 2011. Removal of arsenic from aqueous solution using pottery granules coated with cyst of *Azotobacter* and portland cement: characterization, kinetics and modeling. **Bioresour. Technol., 102:**6308–6312.

[14] Gauri, S.S., S.M.Mandal, K.C.Mondal, S. Dey and B.R. Pati. 2009. Enhanced production and partial characterization of an extracellular polysaccharide of an newly isolated Azotobacter SSB81. **Bioresour Technol., 100:** 4240–4243.

[15] Hay, I.D., Z.U. Rehman, A. Ghafoor and B.H.A. Rehm. 2010. Bacterial biosynthesis of alginates. **J. Chem. Technol. Biotechnol., 85:**752–759.

[16] Hynes,R.K., G.C.Leung, D.L. Hirkala and L.M. Nelson.2008. Isolation, selection, and characterization of beneficial rhizobacteria from pea, lentil and chickpea grown in Western Canada. **Can. J. Microbiol., 54:** 248–258.

[17] Kohler. J. and F. Caravaca. 2010. **An AM fungus and a PGPR intensify the adverse effects of salinity on the stability of rhizosphere soil aggregates of Lactuca sativa Roldan.** *Soil Biol. Biochem.***, 42:**429-434.

[18] Lee,M.C., Y.C.Chen and T.C. Peng. 2012. Two-stage culture method for optimized polysaccharide production in *Spirulina platensis*. **J. Sci .Food Agric., 92:**1562–1569.

[19] Looijesteijn,P.J., L. Trapet, E.de-Vries, T. Abee and J. Hugenholtz. 2001. Physiological function of exopolysaccharides produced by *Lactococcus lactis*. **Int. J. Food Microbiol., 64:**71–80.

[20] Lozano, E., E. Galindo and C.F. Pena. 2011. Oxygen transfer rate during the production of alginate by *Azotobacter vinelandii* under oxygen limited and non

oxygen-limited conditions. **Microb. Cell Factories., 10:**13.

[21] Mandal, S.M., B.R. Pati, A.K.Das and A.K. Ghosh. 2008. Characterization of a symbiotically effective *Rhizobium* resistant to arsenic: Isolated from the root nodules of *Vigna mungo* (L.) Hepper grown in an arsenic-contaminated field. **J. Gen. Appl. Microbiol., 54:**93–99.

[22] Maqubela,M.P., P.N.S.MnkenI, O.Malam issa, M.T.Pardo, and L.P.Dacqui. 2009. *Nostoc* cyanobacterial inoculation in South African agricultural soils enhances soil structure, fertility, and maize growth. **Plant Soil., 315:**79-92.

[23] Muthuvel, P. and C. Udayasooriyan. 1997. Determination of bulk density and pore space. In**: soil, water and chemical analysis**. Tamil Nadu Agricultural university, Coimbatore, pp.81-82.

[24] Ramasawmy, K., V.Gomathy and R. Subramanian.1992. Isolation and characterization of microbial products involved in stable soil aggregation. **In:** Final report submitted to ICAR, Adhoc Research Project Dept. of environmental sciences, TamilNadu Agrl. University, Coimbatore, India.p 115.

[25] Rehm, B.H.A. 2010. Bacterial polymers: biosynthesis, modifications and applications. **Nat Rev Microbiol 8:**578–592.

[26] Segura, D., J. Guzmaan and G. Espin.2003. *Azotobacter vinelandii* mutants that overproduce poly-beta-hydroxybutyrate or alginate. **Appl. Microbiol. Biotechnol., 63:**159–163

[27] Somers, E., J. Vanderleyden and M. Srinivasan. 2004. Rhizosphere bacterial signalling: a love parade beneath our feet. **Crit. Rev. Microbiol., 30:**205–240.

[28] Soussi, M., M. Santamaria, A. Ocana and C. Lluch. 2001. Effects of salinity on protein and lipopolysaccharide pattern in a salt-tolerant strain of *Mesorhizobium ciceri*. **J. Appl. Microbiol., 90:**476-481.

[29] Tank, N. and M. Saraf. 2010. Salinity-resistant plant growth promoting rhizobacteria ameliorates sodium chloride stress on tomato plants. **J. Plant Interact., 5:** 51–58.

[30] Tikhonovich, I.A. and N.A. Provorvo. 2011. Microbiology is the basis of sustainable agriculture: an opinion. **Ann. Appl. Biol., 159:** 155–168.

[31] Vanderlinde, E.M., A. Muszynski, J.J. Harrison, S.F. Koval, D.L.Foreman, H. Ceri, E.L. Kannenberg, R.W. Carlson and C.K.Yost. 2009. *Rhizobium leguminosarum* biovar viciae 3841, deficient en 27-hydroxyoctacosanoate-modified lipopolysaccharide, is impaired in dessication tolerance, biofilm formation and motility. **Microbiol. SGM., 155:**3055-3069.

[32] Vanderlinde, E.M., J.J. Harrison, A. Muszynski, R.W. Carlson, R.J. Turner and C.K. Yost. 2010. Identification of a novel ABC-transporter required for desiccation tolerance and biofilm formation in *Rhizobium leguminosarum* bv. *viciae* 3841. FEMS **Microbiol. Ecol., 71:** 327–340.

[33] Yao. L., Z. Wu, Y. Zheng, I. Kaleem and C. Li. 2010. **Growth promotion and protection against salt stress by Pseudomonas putida Rs-198 on cotton.** *European J. Soil Biol* **., 46:**49-54.