Characterization and phylogenetic relatedness of *Azotobacter Salinestris*

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**ABSTRACT**

With an objective of identifying the free living Nitrogen fixing bacterial diversity from the soils of North Gujarat area, a total 35 free living nitrogen fixing bacterial isolates from different regions of North Gujarat were isolated. Pure cultures were obtained on selective media. One individual isolate was selected for the molecular characterization and phylogenetic relatedness. The biochemical and molecular characterization of the isolate was carried out. The bacterial isolate was identified as *Azotobacter salinestris*. The phylogenetic tree was constructed and it showed strong homology of the isolate with *Azotobacter salinestris*.

**Keywords:** Biological N\(_2\) fixation, *Azotobacter salinestris*, phylogenetic tree

**INTRODUCTION**

After water, nitrogen is most often the limiting factor for plant growth [1]. Crops such as wheat, rice, and maize need 20 to 40 kg soil N ha\(^{-1}\) over a period of 3 to 5 months to satisfy the N requirements for each tonne of grain produced [2]. To meet such high demand, farmers must either apply inorganic synthetic N fertilizers to their land or rely on biological nitrogen fixation (BNF) and the input of recycled organic wastes, such as manure. Adding nitrogen in the form of synthetic fertilizers can have negative environmental impacts since inorganic N, particularly nitrate, can be dispersed into surface and groundwater, leading to eutrophication [3]. In addition, the manufacture of N fertilizers relies on nonrenewable fossil fuels (the production of 1 kg N fertilizer requires 38,000 kJ of fossil energy) [4] and results in significant emissions of greenhouse gases [5]. These environmental concerns, coupled with increasing fuel costs and a desire for improved sustainability have led some farmers to seek alternative N management strategies [6]. N cycling in natural ecosystems and traditional agricultural production relies on biological N fixation primarily by diazo- trophic bacteria. Diazotrophs are highly diverse and are widely distributed across bacterial and archaeal taxa [7]. Most (80%) of biological nitrogen fixation (BNF) is carried out by diazotrophs in symbiosis with legumes [8]. However, underspecific conditions bacteria which are free-living in soil (e.g., cyanobacteria, Pseudomonas, Azospirillum, and Azotobacter) may fix significant amounts of nitrogen (0 to 60 kg N ha\(^{-1}\)year\(^{-1}\)) [9, 7]. This may be particularly important in organically managed soils, which typically have a lower proportion of nitrogen in available forms [10]. The effects of crop management on diversity and function of the soil microbial community are equivocal. Many authors report an increase in total biomass and microbial activity when organic matter inputs are increased and chemical amendments are reduced [11, 12, 13]. In contrast, Donnison et al. [9] found that a change in management had no effect on soil nutrient status, soil microbial biomass, and soil microbial activity. However, they did find that management practices significantly affected the soil microbial community structure and suggested that this was due to changes in plant composition and the form and quantity of fertilizer applied [8]. Diazotrophic community structure and diversity have been shown to respond to changes in grazing, liming, the nature of the nitrogen added, and incorporations of crop residues [14, 11, 15]. They are also especially sensitive to chemical inputs, such as pesticides [12]. The nitrogenase enzyme catalyzes the reduction of atmospheric dinitrogen to ammonia. This process is very energy expensive and is, therefore, tightly regulated [16]. At neutral pH, low levels of fixed N and increased levels of C will allow more optimal conditions for free-living N.
fixation [17]. These conditions are more likely to be found in organically managed soils as increased organic C is added in the form of manure and on average less readily available nitrogen is applied.

Azotobacters are gram–negative, nitrogen–fixing soil bacteria that have extremely high respiration rates. Azotobacter can fix at least 10 mg nitrogen per gram of carbohydrate [18]. This bacterium is an obligate aerobic, although it can grow under low pO2. Nitrogen fixation is achieved by the enzyme nitrogenase, which reduces N₂ to ammonia. However, this enzyme is extremely sensitive to oxygen in Azotobacter species. High respiration rates and conformational protection of the enzyme are suggested as two factors which make nitrogen fixation possible in an aerobic environment [19]. Reduction of O₂ by Azotobacter species occur at such a high rate that large amounts of superoxide radicals are produced [20]. Hammad (1998), reported that Azotobacter is a free-living N₂ fixing bacterium and related to soil organic components, and the amount of nitrogen fixation is lower in Azotobacter compared to the associative and symbiotic bacteria [21].

MATERIALS AND METHODS

2.1 Sample collection and isolation
Rhizospheric soil at a depth of 0 to 15 cm was sampled from different area from Sabarkantha, Banaskantha, Patan and Mehsana districts of North Gujarat during 2010. The samples were brought to the laboratory for analysis. Each 10 gm of soil sample was added to 200 ml of sterile distilled water and soil dispersing agent was added in the flask. The flasks were kept on rotary shaker for one hour. Then serial dilutions were prepared up to 10⁻⁸ to get isolated colonies. The soil suspensions were streaked on sterile Ashby’s Mannitol agar medium and plates were incubated in incubator at 30 °C for a week. Well-developed and separated colonies from the surface of Ashby’s Mannitol Agar medium were each picked up with a sterile needle and suspension was prepared and Gram staining was performed. Results of Gram staining were noted down. Each isolate was transferred separately on Ashby’s Mannitol Agar slant. Each of these new slant cultures represents the growth of a single bacterial species. The colonies, which were different in appearances and characters were picked and purified.

2.2 Characterization and Identification of the isolate.

2.2.1 Morphological and physiological characterization
Strains were characterized by cell (Gram stain, determination of the presence of spores), colony (form, elevation, margin, appearance, optical property, pigmentation, texture), morphological and cultural characteristics. Biochemical tests (gelatin liquefaction, Lipase production, starch hydrolysis,) and sugar utilization tests (glucose, lactose, Sucrose, Mannitol, Xylose, Galactose), oxidase test, nitrate reduction test, Ammonia production test, H₂S production, Oxidase, catalase test were performed [22,23]

2.3.2 Genetic analysis

2.2.2.1 Genomic DNA extraction from isolate
Genomic DNA was extracted from the isolate using a MEDOX-Bio™ Ultra pure Genomic DNA Spin Miniprep kit, following the manufacturer’s instructions. The cell pellet was re-suspended in 200 µl cold TE buffer. 400 µl of Digestion solution was added, mixed and 3 µl of proteinase K solution was added and incubated for 5 minutes. Then 260 µl of ethanol was added and thoroughly mixed. The mixture was applied to column placed in 2 ml of collection tube. The mixture was then centrifuged at 8000 rpm for 1 minute. 500 µl of wash solution was added and spin at 8000 rpm for 1 minute. This step was repeated. The flow through and collection tube was discarded. The mini spin column was carefully removed and placed in 1.5 ml microcentrifuge tube, 30µl of elution buffer was added and was incubated for 2 minutes at room temperature and then centrifuged at 10000 rpm for 1 minute. The DNA sample was then stored at -20°c and thawed at room temperature as required.

2.2.2.2 Amplification of 16S r-DNA by polymerase chain reaction (PCR)
In PCR the isolated DNA was amplified by using 16S r-DNA primers (F- 5’ AGAGTRTGATCMYTGYCTWCAC-3’ 5’-CGYTAMCTTWTTACGRT-3’) The quantity of reagents added and parameters maintained in PCR are as follows: DNA: 1µl,16s Forward Primer 400ng 16s Reverse Primer 400ng, dNTPs (2.5mM each) 4µl, 10X Taq DNA Polymerase, Assay Buffer 10µl, Taq DNA Polymerase Enzyne (3U/µl) -1µl, D.Water 84 µl, Total reaction volume-100µl. The DNA was amplified by using Thermal cycler the parameters set up were Initial denaturarion temp- 94°C -05 min, Denaturation temp.94°C-30 sec. Annealing temp 55°C-30 sec, extension temp.-72°C -02 min, Final extension temp 72°C.- 5 min for 35 cycles. PCR Product was observed by using 500bp ladder marker.
2.2.2.3 DNA sequencing and identification
The sequencing of PCR Product was carried out by using ABI 3500 XL Genetic Analyzer at Chromus Biotech Pvt. Limited, India. The similarity of sequence of the isolate was aligned by using a bioinformatics tool BLAST. The alignment view and distance matrix table was created. The sequence of the identified isolate was sent to NCBI.

2.2.2.4 Phylogenetic analysis
Phylogenetic Tree Builder uses sequences aligned with System Software aligner. A distance matrix was generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions were used, alignment inserts were ignored and the minimum comparable position was 200. The tree was created using Weightor with alphabet size 4 and length size 1000

RESULTS AND DISCUSSION
Considering cell morphological characteristics, isolated organism was found to be Gram negative and do not form endospore. On N₂ free Ashby’s Mannitol Agar medium the colonies were gummy and grows up to 1-2 mm in diameter after seven days of incubation. The shape of the colonies was round, margins were entire, the surface of the colonies were glistening. Most frequently the density of colonies in the middle is opaque and in the margins was transparent. The organism was heterotrophic bacteria present the property of fermenting glucose, manniitol, xylose
and sucrose producing acids, but did not utilize lactose, galactose and maltose. The Biochemical characterization performed by bacteria shown in Table-1 and Table 2

Table 1 Selected biochemical tests of isolate after 48 hours of incubation at 30°C±0.5°C.

<table>
<thead>
<tr>
<th>Name of the Test</th>
<th>Response of strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂S production</td>
<td>-</td>
</tr>
<tr>
<td>Indole production</td>
<td>-</td>
</tr>
<tr>
<td>Methyl Red reaction</td>
<td>-</td>
</tr>
<tr>
<td>Voges-Proskauer reaction</td>
<td>-</td>
</tr>
<tr>
<td>Citrate Utilization</td>
<td>-</td>
</tr>
<tr>
<td>Urease activity</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>++</td>
</tr>
<tr>
<td>Nitrate Reduction test</td>
<td>++</td>
</tr>
<tr>
<td>Ammonia production</td>
<td>++</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Lipid hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 2 Selected carbohydrate utilization tests of isolate after 48 hours of incubation at 30°C±0.5°C.

<table>
<thead>
<tr>
<th>Carbohydrate Utilization test</th>
<th>Response of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>+++</td>
</tr>
<tr>
<td>Maltose</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+++</td>
</tr>
<tr>
<td>Mannose</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>++</td>
</tr>
<tr>
<td>Xylose</td>
<td>++</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
</tr>
</tbody>
</table>

According to genetic analysis the amplified 16Sr-DNA sequence of the isolate gave 1.5kb fragment. By using sequence of bacterial isolate taken as a reference sequence Alignment view and distance matrix table which shows the similarity with the *Azotobacter salmonicola* is listed in Table 3. The sequence was submitted to NCBI and the accession number provided was JX437935. The phylogenetic tree was constructed as shown in fig-3 and the position of isolate and relatedness with other bacteria was determined. The result of phylogenetic tree analysis shows that the isolate JP-16 lies between *Azotobacter salinestris* I-A and *Azotobacter vinelandii*. However the isolate JP-16 shows 94% homology with *Azotobacter salinestris* I-A than *A. vinelandii* which is 90%

Table 3

ALIGNMENT VIEW and DISTANCE MATRIX TABLE:
(With Sample JP-16-sequence taken as reference sequence)

<table>
<thead>
<tr>
<th>S_ab score</th>
<th>Organism Name</th>
<th>NCBI Accession No</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.992</td>
<td><em>Azotobacter salmonicola</em> (T); ATCC 49674</td>
<td>AR175656</td>
</tr>
<tr>
<td>0.991</td>
<td><em>Azotobacter vinelandii</em>; ISSDS-379</td>
<td>EF620433</td>
</tr>
<tr>
<td>0.992</td>
<td><em>Azotobacter vinelandii</em>; ISSDS-380</td>
<td>EF620434</td>
</tr>
<tr>
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<td>EF620436</td>
</tr>
<tr>
<td>0.992</td>
<td><em>Azotobacter vinelandii</em>; ISSDS-384</td>
<td>EF620437</td>
</tr>
<tr>
<td>0.992</td>
<td><em>Azotobacter vinelandii</em>; ISSDS-385</td>
<td>EF620438</td>
</tr>
<tr>
<td>0.992</td>
<td><em>Azotobacter salinestris</em> I-A</td>
<td>FJ032010</td>
</tr>
<tr>
<td>0.992</td>
<td><em>Azotobacter vinelandii</em>; FY10</td>
<td>FJ797400</td>
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<tr>
<td>0.992</td>
<td><em>Azotobacter vinelandii</em>; M2Per</td>
<td>GQ204712</td>
</tr>
</tbody>
</table>
CONCLUSION

Many various strains were found during the isolation and characterization of bacteria from soil of North Gujarat. The phylogenetic tree of the isolate shows its relatedness with *Azotobacter* genus. The position represents identification of many species and between these we can found some new species with biotechnological and ecological agricultural importance. The significance of our research consists in the examination for the efficient free living nitrogen fixer first time in soils of North Gujarat.

REFERENCES