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Occurrence of *Azotobacter chroococcum* in Wheat rhizosphere collected from Aurangabad District of Maharashtra

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ABSTRACT

Azotobacter is a non-symbiotic free living nitrogen fixing bacteria which is generally used as Plant Growth Promoting Rhizobacteria (PGPR). A critical survey has carried out to collect rhizospheric soil samples from various localities of Aurangabad district. Total 27 soil samples were selected from Wheat rhizosphere and isolated on Azotobacter Manitol Agar (HiMedia- M372). Biochemical tests like starch hydrolysis, catalase, citrate, indole production, urease and nitrate reduction were performed for primary identification of bacterial samples. The advance technique for confirmation of bacterial samples were used i.e.16s rRNA sequencing. Obtained gene sequences were compared to those of the most closely related bacterial species using the NCBI BLAST program. Present investigation is the first report from Aurangabad district describing morphological, biochemical and molecular characterization of A. chroococcum collected from wheat rhizosphere.

KEYWORDS: PGPR, Azotobacter, 16s rRNA, Aurangabad district.

1. INTRODUCTION

Azotobacter represent the main group of heterotrophic, non-symbiotic free living nitrogen fixing bacteria. Which was used as bio inoculant for various crops [9]. pH is main factor for controlling the colonisation of *Azotobacter* in soil [8]. Various workers concluded that high correlation coefficients between reaction (pH) of the tested soils and the occurrence of *Azotobacter* spp. in soils confirm the well-known sensitivity of these bacteria to low soil pH [CR 2, 4, 16 and 17] [7].

Azotobacter is a free-living aerobic bacteria dominantly found in most of the soils or in rhizosphere of many crop plants. The well-known species of *Azotobacter* are *A. chroococcum*, *A. agilis*, *A. vinelandii*, *A. beijerinckii*, *A. insignis*, *A. macrocytogenes*, *A. paspali* etc. *Azotobacter* can fix atmospheric nitrogen and improve soil fertility without symbiotic association with plant [6].

The ecological distribution of *Azotobacter* spp. is a complicated and is related with diverse factors which determine the presence or absence of this bacterium in a specific soil [14]. It has been indicate the soil characteristics. Environment circumstances affect the distribution of *Azotobacter* species. The *Azotobacteraceae* are belong to the genera, *viz.*, *Azotobacter*, *Beijerinckin* and *Erexia*. *Azotobacter chroococcum* efficiently increases the growth and yields of crops in refined soils having high organic matter content, and nitrogen fixation. *Azotobacter* is also known to synthesize biologically active substances

such as B-Vitamins, indole acetic acid and Gibberellins in pure cultures. The organism also possesses fungicidal properties even on certain pathogenic ones such as *Alternaria* and *Fusarium*. These characteristics of *Azotobacter* helps in improving seed germination and plant growth [13].

2. MATERIALS AND METHODS

1. Soil sample collection: rhizospheric soil sample were collected from various localities of Aurangabad district. Soil sample collect in sterile zip lock bags from 10-15 cm depth of wheat rhizosphere.

2. Serial dilution of rhizosphere soil samples: 1gm of rhizosphere sample was diluted in 9ml of distilled water. Again 1ml of this solution transferred in serially 5 test tubes and lastly diluted solution was used for further investigation [12].

3. Isolation of Bacteria: Serially diluted solution was used to inoculate on nutrient broth using streak plate method. Then the plates were incubated in incubator for 48 hrs at 25 ± 2^0 c [10]. For confirmation of bacterial culture grown colony further sub-cultured on selective medium *i.e.* Azotobacter Manitol Agar (HiMedia- M372). Then pure cultures were preserved on slants of the same selective media.

4. Bacterial Characterization: Morphological and Biochemical characterization was done by using various scientific methods [11]. The morphological tests contains colony morphology, cell morphology and Gram staining; while biochemical tests contains Starch Hydrolysis, Catalase, Urease, Citrate, Indole Production and Nitrate Reduction.

5. Biochemical tests: Biochemical tests like Starch Hydrolysis, Catalase, Citrate and Indole Production were performed by using the method adopted by Hossain *et al*., [5]. Urease and Nitrate reduction was done as per the method adopted by Phalke *et al*., [11].

6. Molecular characterization: Every bacteria having its unique conserved region on rRNA, hence the advance technique for identification of bacterial samples were used *i.e.*16s rRNA sequencing. Gene sequences were compared to those of the most closely related bacterial species using the NCBI BLAST program (http: //www.ncbi.nlm.nih. gov/BLAST) [1]. Universal 16S PCR primers were selected for *in silico* compression *viz.* 27F, 63F, 519R, 910R, G03F and G07R best sequence were recorded 98 to 100% coverage with G03F and G07R primers hence these primer used for identification of *Azotobacter* spp.

3. RESULT AND DISCUSSION

Total 27 soil samples were collected from Wheat rhizosphere pH of collected soil sample was ranging 7.1 to 7.6 while Water Holding Capacity (WHC) was ranging between 29.32 to 37.46%. (Table 01) *A. chroococcum* is a Gram –ve, rod shaped bacteria forming translucent, oval and cream white coloured colonies with entire margin on special culture media. During the present investigation, average bacterial cell length recorded in Aurangabad district was 4.1µm; while average colony size was 3.9 mm (Table 02). Various biochemical tests were performed for preliminary identification of *A. chroococcum* and the data is presented in table 03. *A. chroococcum* hydrolyses starch and produce abundant amount of indole for promoting growth of the plant. *A. chroococcum* luxuriantly converts Nitrate (NO_3) to Nitrite (NO_2), which is available form of Nitrogen (N_2) for the plant in soil. After the process of denitrification or nitrate reduction by *A. chroococcum* produces Nitrite (NO_2) which becomes the major source of nitrogen as a macronutrient. *A. chroococcum* hydrolyse the urea and convert it into two molecules of ammonium which is another source of nitrogen. *A. chroococcum* utilizes citrate as a source of energy and produces catalase enzyme required to protect the plant cell from oxidative damage.

Obtained partial sequence (1528bp) from bacterial sample code AU49 were search on NCBI BLAST program showed 98% query coverage while 99.89% identity with *A. chroococcum* recorded in the bacterial sample. The analyses of the 16S rRNA regions revealed that the bacterial sample AU49 was *A. chroococcum*. Some of the potential phenotypic differences are still uncovered.

Present investigation is the first report from Aurangabad District, which describes morphological, biochemical and molecular characterization of *A. chroococcum* isolated from rhizosphere of wheat. Occurrence of *A. chroococcum* in wheat rhizosphere shows positive effect on growth of the plant [16]. Dadook *et al* [3] reported that *A. chroococcum* was gram –ve bacteria and shows positive response to nitrate reduction and urease test. Same biochemical and morphological characters of *A. chroococcum* was recorded by Upadhyay *et al* [15]

>*Azotobacter chroococcum* strain DC4 16S ribosomal RNA gene, partial sequence
Sequence ID: MH763851.1 Length: 1528
Range 1: 1 to 876

Score: 1613 bits (873), Expect: 0.0,
Identities: 876/877(99%), Gaps: 1/877(0%), Strand: Plus/Minus

TCCCCAGGCGGTCGACTTAATGCGTTAGCTGCGCC
ACTAAGCTCTCAAGGAGCCCAACGGCTAGTCGAC
ATCGTTTACGGCGTGGACTACCAGGGTATCTAATC
CTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAG
TATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTC
CTTCCTATATCTACGCATTTCACCGCTACACAGGA
AATTCCACCACCCTCTACCGTACTCTAGCCTATATC
TACGCATTTCACCGCTACACAGGAAATTCCACCAC
CCTCTACCGTACTCTAGTCAGGCAGTTTTGGATGC
AGTTCCCAGGTTGAGCCCGGGGCTTTCACATCCAA
CTTACCAAACCACCTACGCGCGCTTTACGCCCAGT
AATTCCGATTAACGCTTGCACCCTTCGTATTACCGC
GGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTG
TCGGTAACGTCAAAACTGCAAGGTATTCGCTTACA
GCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCG
AAGACCTTCTTCACACACGCGGCATGGCTGGATCA
GGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGC
CTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAG
TGTGACTGATCATCCTCTCAGACCAGTTACGGATC
GTCGCCTTGGTGAGCCTTTACCCCACCAACTAGCT
AATCCGACCTAGGCTCATCCGATAGCGTGAGGTCC
GAAGAGCCCCCACTTTCTCCCGTAGGACGTATGCG
GTATTAGCGTTCCTTTCGAAACGTTGTCCCCCACTA
TCGGGCAGATTCCTAGGCATTACTCACCCGTCCGC
CGCTGAATCGGGATGCAAGCACCCCTCATCCGCTC
GACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAA
TCTGAGCCAGGATCAAACTCT

Table 01: Sample Collection from Aurangabad District

Sample Code	Location	Rhizosphere of the crop	Physical Properties of Soil		
			Soil Type	pH	Water Holding capacity
KA01	Lamangaon (West Side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.5	29.85 %
KA16	Auroli (East Side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.2	32.56 %
KA25	Dongaon (East Side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.6	31.34 %
KH02	Khultabad (Hill Side)	*Triticum aestivum* (Wheat)	Rocky soil	7.5	29.97 %
KH10	Kasabkheda	*Triticum aestivum* (Wheat)	Black soil	7.5	36.64 %
KH21	Gadana (West Side)	*Triticum aestivum* (Wheat)	Rocky Soil	7.4	31.62 %
KH26	Viramgaon (South Side)	*Triticum aestivum* (Wheat)	Light Black Soil	7.3	35.46 %
PA01	Dhorkin (North Side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.1	32.45 %
PA04	Paithan (North Side)	*Triticum aestivum* (Wheat)	Black cotton soil	7.4	32.46 %
PA07	Dhupkheda (West Side)	*Triticum aestivum* (Wheat)	Loamy soil	7.3	35.46 %

PA16	Pachod (West Side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.2	34.56 %
PA22	Shringarwadi (North Side)	*Triticum aestivum* (Wheat)	Black cotton soil	7.3	36.57 %
PH04	Jategaon (West side)	*Triticum aestivum* (Wheat)	Black soil	7.6	37.46 %
PH09	Shelgaon (West side)	*Triticum aestivum* (Wheat)	Rocky soil	7.2	29.32 %
PH17	Phulambri (West side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.4	35.54%
PH21	Vaghalgaon (West side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.4	36.58 %
SI08	Undangaon (North Side)	*Triticum aestivum* (Wheat)	Light Black Soil	7.2	36.58 %
SI15	Talvada (South Side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.4	34.87 %
SI20	Chinchkheda (West Side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.5	35.55 %
SI23	Kelgaon (South Side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.3	34.66 %
SO01	Jawala (East Side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.3	34.65 %
SO09	Nandgaon (West Side)	*Triticum aestivum* (Wheat)	Light black soil	7.5	35.64 %
SO16	Kaldari (East Side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.1	34.56 %
VA04	Vaijapur (West Side)	*Triticum aestivum* (Wheat)	Black soil	7.3	36.20 %
VA12	Palkhed (West Side)	*Triticum aestivum* (Wheat)	Grey water drainable soil	7.2	36.41 %
VA19	Nagamthan (North Side)	*Triticum aestivum* (Wheat)	Black soil	7.4	36.97 %
VA25	Satana (East side)	*Triticum aestivum* (Wheat)	Black soil	7.2	37.72 %

Table 02: Morphological characterization of *A. chroococcum*

Sr. No.	Characters	Result

1	Color of colony	Creamy white
2	Shape of colony	Circular
3	Average colony size	4.2 mm
4	Appearance of colony	translucent
5	Margin of colony	Entire
6	Gram's test	Negative
7	Cell shape	Rod

Table 03: Biochemical characterization of *A. chroococcum*

Sr. No.	Biochemical tests	Activity
1	Starch hydrolysis	+ve
2	Catalase	+ve
3	Urease	+ve
4	Citrate	+ve
5	IAA production	+ve
6	Nitrate reduction	+ve

Conflict of interest statement

Authors declare that they do not have any conflict of interest.

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