

Molecular Evolution of the Negative Regulatory Gene (*NIFL*) from *Azotobacter Chroococcum* and its Nitrogenase Activity

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Two isolates of *Azotobacter chroococcum* (*A. chroococcum* CBD15 and *A. chroococcum* W5), were characterized for their atmospheric nitrogen fixing efficiency and ability to produce plant growth promoting hormone. The isolates, *A. chroococcum* CBD15 and *A. chroococcum* W5, were observed the production of Indole acetic acid (IAA) and nitrogen fixation in the absence of any inorganic nitrogen source. The ability nitrogen fixation was estimated by acetylene reduction studies revealed that *A. chroococcum* CBD15 produced 693.3 nmole C₂H₄ h⁻¹ mg⁻¹ whereas *A. chroococcum* W5 produced 523.4 nmole C₂H₄ h⁻¹ mg⁻¹. Nitrogenase activity of both the isolates was reduced when grown in media containing nitrogen source (ammonia or urea), in comparison to media lacking any nitrogen source. Both the isolates were screened for production of IAA, plant growth promoting substance. The *nifL* gene, which is one of the most important regulatory gene of nitrogen fixation pathway, was isolated from *A. chroococcum* CBD15 and *A. chroococcum* W5. Sequence analysis revealed that both *nifL* gene sequences have maximum homology with *nifL* gene of *A. vinelandii* and *Pseudomonas oryzae* respectively. The genetic manipulation of *nifL* gene of *A. chroococcum* will lead to development of an efficient bioinoculant for sustainable agriculture.

Keywords: *Azotobacter chroococcum*, NifL, NifA, Acetylene reduction, IAA.

Nitrogen is the most abundant and inert gas, present in the atmosphere and ‘nitrogen’ element is one of the most essential requirements for healthy plant growth. In natural systems, nitrogen required for plant growth comes from various sources as soil, rainfall, atmospheric deposition or biological nitrogen fixation. Plants can absorb nitrogen only in the form of ammonia or nitrate but the ammonia and nitrate levels are often very low in the soil. Thus, plants face poor nutritional availability with respect to nitrogen,

causing negative effect on yield (Prescott *et al.* 1999). A few prokaryotic organisms have the nitrogen fixing ability ie they convert atmospheric nitrogen to a form that plants can easily uptake it. These “biological nitrogen fixers” are reported to be the largest contributors of nitrogen to soil, in a form that is available to plants (Burriss and Roberts 1993).

Among the prokaryotic nitrogen fixers, the most prominent are the heterotrophic diazotroph present in the rhizosphere of plants. These

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prokaryotes are capable of utilizing atmospheric nitrogen for their own growth; and as products of nitrogen fixation process, they also release soluble forms of nitrogen in the soil (Damir *et al.* 2011; Sayeda *et al.* 2011). They exist as free-living or as symbionts associated with cyanobacteria, various plants including grasses, woody plants and a number of legumes in diverse habitats of soil and water (Dixon and Kahn 2004).

The nitrogen fixing microbes such as *Klebsiella pneumoniae* and *Azotobacter vinelandii* was well studied. Members of the genus *Azotobacter* belong to the family Azotobacteriaceae and are free-living obligately aerobic, nitrogen fixing heterotrophic diazotrophs. They include gram-negative α -proteobacteria and exist as rod-shaped cells varying in size from 2.0-7.0 to 1.0-2.5 μm (length X breadth). *A. chroococcum* was the first species of *Azotobacter* genus to be described followed by other species which include *A. vinelandii*, *A. armeniacus*, *A. paspali*, *A. beijerinckii*, *A. nigricans* and *A. salinestris* (Robert *et al.* 2015).

The complex biological process of nitrogen fixation is governed by a number of enzymatic complexes. One of the key enzymes that crucial role in catalyzing biological nitrogen fixation is nitrogenase. Nitrogenase is a metalloenzyme that subdivides two components, which called their related to presence of metal irons such as Fe-protein and MoFe-protein (Dixon and Kahn 2004). Iron (Fe) protein plays important role in ATP-dependent electron donor to the larger component. It contains covalently linked two α subunits of metal cluster ([4Fe-4S] cluster). The [4Fe-4S] cluster involved in electron transfer to FeMo-protein which cycles between reduced and oxidized state (Igarashi and Seefeldt 2003). The MoFe protein, contains the enzyme catalytic site (Rees *et al.* 2005). The group of genes responsible for biological nitrogen fixation is together termed as 'nitrogen fixation genes' or *nif* genes. So far, twenty one *nif* genes have been reported from different nitrogen fixing bacteria. All the reports demonstrated that minimum of six conserved genes: *nifH*, *nifD*, *nifK*, *nifE*, *nifN*, and *nifB* genes was found in all diazotrophs for nitrogen fixation. The genes responsible for regulation of nitrogen fixation, *nifH*, *nifD* and *nifK* are considered as the most important *nif* genes of the *nif* operon as they together code for the metalloenzyme

called nitrogenase. Nitrogenase plays a pivotal role in catalyzing biological nitrogen fixation along with the gene *nifA*, the positive regulator of the *nif* operon.

The *NifA* is an activator and positive regulator-encoding gene that binds to specific upstream gene sequence of *nif* genes to initiate transcription by interacting with σ^{54} subunit of the RNA polymerase. The expression of the gene *nifA* leads to expression of genes related to nitrogenase biosynthesis including the structural gene cassette *nifHDK*. Molecular biology techniques are been utilized for identification of nitrogen fixing bacteria based on the presence of structural gene (*nifHDK*) and other genetic elements. Sequence analysis of *nifH* gene has shown that like all other *nif* genes, *nifH* is also highly conserved across all nitrogen-fixing organisms indicating that this gene has not undergone many changes through evolutionary process (Zehr *et al.* 2003). Gene *nifL* acts as a negative regulator of the *nif* operon, in the presence of inorganic nitrogenous source as ammonia (fixed Nitrogen) (Oliveira *et al.*, 2012). Although there has been immense study on different biological nitrogen fixers yet we still need to identify an efficient microbial nitrogen fixer based on geographical location, agroclimatic zone and associative crop. Moreover, intensive molecular biology approaches are needed to understand and rectify the negative regulation of gene *nifL* (Bageshwar *et al.*, 2017).

The present study aimed at assessing the nitrogen fixing and growth promoting activity of two native isolates of *A. chroococcum* (*A. chroococcum* CBD15 and *A. chroococcum* W5) for their utilization as bioinoculants for enhanced nitrogen availability in soil for crop plants. Further, with the aim of manipulating the *nifL* gene for deleting its negative regulation ability, the *nifL* gene from both the isolates, *A. chroococcum* CBD15 and *A. chroococcum* W5 were sequenced and characterized.

MATERIALS AND METHODS

Azotobacter Strains and Growth Medium

Azotobacter isolates (*A. chroococcum* CBD15 and *A. chroococcum* W5) were collected from Division of Microbiology, Indian Agricultural Research Institute, New Delhi (Paul and Verma

1999; Paul *et al.* 2011). The strains were further sub-cultured on Burk's medium (Kennedy *et al.* 1986). The purity of the cultures was checked by streak plate method and maintained on the slants of respective medium at 4°C in a refrigerator for medium term storage.

Molecular Characterization of *nifL*

Extraction of genomic DNA from the *A. chroococcum* isolates CBD15 and W5 was carried out by manual method (George *et al.*, 2007). The overnight cultures (OD= 1.2) were harvested and the pellet was washed with 0.7% NaCl. The pellet consisting of bacterial cells was treated with lysis solution, containing Tris (100mM), EDTA (10mM) and SDS (0.1%) and Proteinase K (20mg/l). The lysate was incubated at 65°C for 30 minutes to release nucleic acid. Following its release-proteins and cellular debris removed by precipitation and chloroform:isoamyl alcohol (24:1) separation. The nucleic acid was then recovered from the clarified lysate by adding 0.7 volume of isopropanol. The purity of the nucleic acid was determined by measuring its optical density at 260nm and 280nm using Nano drop (Nanodrop 6000, ThermoFisher). The *nifL* gene was amplified in *A. chroococcum* isolates CBD15 and W5 through polymerase chain reaction (PCR) using gene specific primers (forward F 5'-ATCGCGCTTCCGCACCATCACCG-3'; reverse R 5'- TCGACTCTAGAGGTG AGGCCGA-3') and genomic DNA of these isolates as template. The primers were designed based on sequence available in the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>). The PCR reaction mix contained in a final volume of 50µl: 5µl buffer (Genei), 1.5µl MgCl₂ (50mM), 5µl dNTP-mix (2.5mM each), 1µl each primer (10µM), 10 ng purified DNA of cultured strains and 1U Taq-DNA polymerase (Genei). The PCR was performed with initial denaturation for 5minutes at 94°C, 35 cycles with 30s 94°C, 30s 55°C, 1 minute at 72°C, and final elongation for 5 minutes at 72°C. The obtained amplicons of PCR were separated by 1.5% agarose gel electrophoresis. The amplified PCR product were cloned in pGEM-T Easy vector (Promega, USA) as per manufacturer's protocol and sequenced and the obtained nucleotide sequences were determined with NCBI BLAST database (<https://blast.ncbi.nlm.nih.gov/>).

Sequence Alignment and Phylogenetic Analysis

The amplicon of ~ 1.4kb was obtained by PCR reaction. The resulting 1.4kb nucleotides sequence were BLAST searched. The new sequences were analyzed with those in the databases by using the basic local alignment search tool (BLAST; <http://ncbi.nih.gov/cgi-bin/nph-blast?Jform=1>). The sequences were aligned with selected genera with the CLUSTAL W program. The phylogenetic tree was analyzed with known sequence from NCBI gene bank from the neighbor-joining method with MEGA version 6.0 (Tamura *et al.*, 2013). *nifL* sequence of *A. chroococcum* isolate CBD15 and *A. chroococcum* isolate W5 were submitted to GenBank to get the accession numbers.

Nitrogenase Activity Assay

Nitrogenase activity of the *Azotobacter* isolates was measured by acetylene reduction assay (Dilworth, 1996). The isolates were inoculated in 5 ml of media and closed with rubber stoppers. The 5ml of air was replaced with 5 ml of acetylene gas (C₂H₂) using a sterile syringe and incubated for 24 hours at temperatures ranging between 28°C and 30°C. Following this standing period, 0.2 ml of gas injected into gas chromatography column to verify the amount of C₂H₂ converted to ethylene (C₂H₄) in each vial. To determine the amount of nitrogenase activity were following the formula used:

$$N = (hx \times C \times V) / (hs \times 24.9 \times t \times \text{mg protein})$$

Where, N = the concentration of C₂H₄ (nmol ml⁻¹ h⁻¹); hx = sample peak value; C = standard concentration of C₂H₄ (nmol ml⁻¹h⁻¹); V = volume of the vial; hs = standard peak value; t = reaction time (h).

The concentration of bacterial protein was determined by Lowry method (Lowry *et al.*, 1951). To 0.2 ml of digested bacterial cell suspension was added to a total volume of 1 ml followed by addition of 5 ml of reagent "C" (copper sulphate-sodium-potassium tartrate solution) and immediate vortexing. Subsequently, the bacterial cell suspension was kept for 10 minutes at room temperature, and added 0.5 ml of reagent "D" (alkaline copper sulphate) and subsequently kept for incubation in the dark for 30 minutes. After 30 minutes of incubation, optical density of the reaction mixture was measured at 660nm and the amount of protein was determined with known

concentration of standard protein (Bovine serum albumin).

Ammonia and Urea Sensitivity Assay

To assess the sensitivity of the *Azotobacter* strains to ammonia, the overnight cultures were inoculated in different media: Burk's medium (Bark, 1930) Burk's medium+ ammonia (0.11%); Burk's medium + 0.1% with urea. The cultures were incubated at 30°C for 48 hours with 200rpm and the OD at 600 nm of culture was measured.

Indole Acetic Acid (IAA) Production Assay

The isolates, *A. chroococcum* CBD15 and *A. chroococcum* W5 were estimated the production of Indole Acetic Acid (IAA) using the protocol given by Bric *et al.* (1991). *A. chroococcum* isolates were inoculated in 100ml of 'Nitrogen free' media ie medium devoid of any nitrogen source. Another set of inoculations of *A. chroococcum* isolates was carried out in 100 ml of 'Nitrogen free' supplemented with tryptophan (100 µg ml⁻¹). All the cultures were incubated at 30°C with shaking of 120 rpm (Kuhner LT-X (Lab-Therm) Switzerland) in for seven days. Subsequently, 2 ml of the liquid culture was transferred to centrifuge tube and centrifuged at 10,000 rmp (Eppendorf™ Model 5810R, Germany) for 15 minutes. The supernatant (1ml) transferred to a fresh centrifuge tube and mixed with 100 µl orthophosphoric acid and Salkowski's reagent (1 ml of 0.5 M FeCl₃ in 50 ml of 35% HClO₄) each and incubated up to colour formation. The colour absorbance was measured spectrophotometrically at 530 nm.

Statistical Analysis

All the experimental results were subjected to analysis of variance (ANOVA) and significance at 5% level was tested by Least Significant Difference (LSD) using SAS package, Version 8.2 (SAS version 8.2, 2001). (<https://support.sas.com/downloads/browse.htm?cat=84>).

RESULTS

Molecular Characterization of *nifL*

The DNA was isolated from *A. chroococcum* CBD15 and *A. chroococcum* W5 cultures and PCR carried out for gene specific amplification of *nifL* gene using primers designed based DNA sequence of *A. vinelandii* gene *nifL* obtained from NCBI database (<https://blast.ncbi.nlm.nih.gov/>; Fig.1) The *nifL* amplicons of

~1.4kb obtained in both the isolates were cloned in pGEMT vector and sequenced. The sequence analysis revealed that amplicons of 1416bp and 1303bp were obtained in *A. chroococcum* CBD15 and *A. chroococcum* W5 respectively which was confirmed as partial sequence of *nifL* gene.

Sequence Alignment and Phylogenetic Analysis

Nucleotide sequence analysis of isolates *A. chroococcum* CBD15 and *A. chroococcum* W5 using ClustalW program revealed that both the isolates showed maximum homology (98%) with *A. vinelandii* and *Pseudomonas oryzae* (Fig. 2). An unknown sequence profile was constructed from the conserved region of negative regulatory gene from *A. chroococcum* isolates CBD15 and W5 with the identical *nifL* gene sequence from NCBI data base using ClustalW program. The results shows that *nifL* gene of *A. chroococcum* CBD15 and *A. chroococcum* W5 were highly similar in sequence (99%, W5:0.00000, CBD15:0.00073) (Fig.2a). Similarity analysis of gene *nifL* of *A. chroococcum* CBD15 and *A. chroococcum* W5 with gene *nifL* of *A. vinelandii* (X64832.1) and *A. chroococcum* (CP010415.1) shows that percent identity matrix was 0.04054 (CP010415.1) and 0.00160 (X64832.1) Yellow and Green color highlighted (Fig 2a).

GenBank/EMBL Accession Number

Accession number for *nifL* sequence of *A. chroococcum* isolates CBD15 and *A. chroococcum* isolates W5 was KY781893 and MF375754 respectively.

Nitrogenase Activity Assay

The nitrogenase activity of *A. chroococcum* CBD15 was found to be the highest under aerobic conditions as compared to the *A. chroococcum* W5 with respect of all three different medium compositions used in the study (Fig. 3). The highest nitrogenase activity of *A. chroococcum* CBD15 was found in media devoid of any nitrogen source (693.3 nmole C₂H₄ h⁻¹ mg⁻¹), However, in media containing 0.1% urea, there was a decrease in nitrogen fixed (365.0 nmole C₂H₄ h⁻¹ mg⁻¹). With 0.11% of ammonium acetate as nitrogen source in the media, nitrogen fixation ability of *A. chroococcum* CBD15 further reduced (213.2 nmole C₂H₄ h⁻¹ mg⁻¹) (Fig. 3). Similarly in the case of *A. chroococcum* W5 nitrogenase activity was found to be higher in media without nitrogen source (523.4 nmole C₂H₄ h⁻¹ mg⁻¹) and decreased with increase in

nitrogenous source (0.1% urea :354.9 nmole C₂H₄ h⁻¹ mg⁻¹; 0.11% ammonium acetate: 204.4 nmole C₂H₄ h⁻¹ mg⁻¹) (Fig. 3).

Ammonia and Urea Sensitivity Assay

The growth characteristics of isolates *A. chroococcum* CBD15 and *A. chroococcum* W5 were determined. The isolates were grown in two different media having different supplementation of nitrogen sources (0.1% urea and 0.11% ammonium acetate). The growth of *A. chroococcum* CBD15

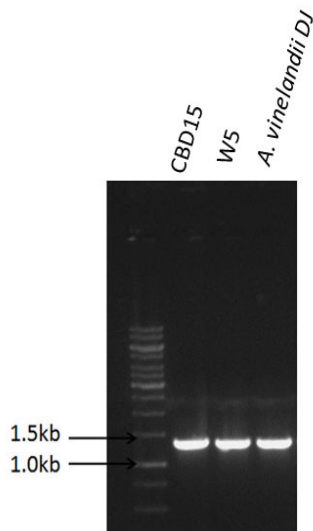


Fig. 1. Negative regulatory gene (*nifL*) amplification with specific primer from *A. vinelandii* sequence data base, *A. chroococcum* CBD15, *A. chroococcum* W5 and reference strain *A. vinelandii* DJ

was observed to gradually increase in media containing 0.11% ammonium as was evident by OD at 600nm of the culture which increased from 0.23 to 0.66 in 48 hours. However, no changes were observed in the growth of *A. chroococcum* CBD15 in medium devoid of any nitrogen source, even after 48 hours. It was observed that in media with 0.1% urea, the growth of culture was very rapid during initial period as evident by the increase in OD at 600nm from 1.45 to 1.56 within 24 hours, however, as the time interval lengthened; the OD at 600nm of the culture began to decrease from 1.56 to 1.12 in 48 hours. Similarly observations were also made in case of growth of *A. chroococcum* W5 in media supplemented with different nitrogen sources. The growth of *A. chroococcum* W5 gradually increased in media containing 0.11% ammonium as was evident by OD at 600nm of the culture which increased from 0.23 to 0.66 in 48 hours as it was in case of *A. chroococcum* CBD15. With 0.1% urea in the media, the growth of culture was very rapid during initial period as evident by the increase in OD at 600nm from 1.34 to 1.78 within 24 hours, however, as the time interval lengthened; the OD at 600nm of the culture began to decrease from 1.42 to 1.18 in 48 hours (Fig. 4).

Indole Acetic Acid (IAA) Production Assay

The IAA production was measured depending on growth phase of bacterial isolates and maximal production of IAA was found in stationary phase. Since presence of tryptophan is necessary for IAA production, the optimum

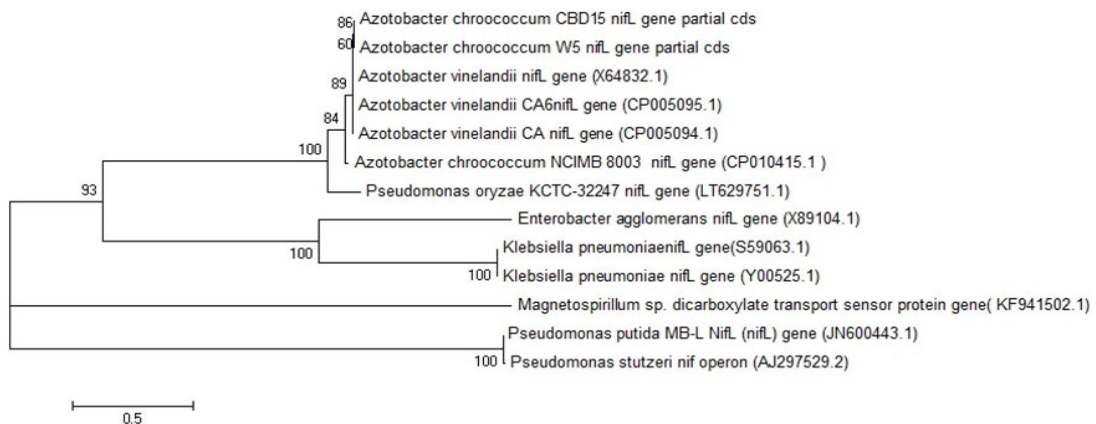


Fig. 2. Phylogenetic trees for Negative regulatory gene (*nifL*) showing the phylogenetic position of *A. chroococcum* among all known corresponding sequences of various diazotrophs. Trees were constructed by the neighbour-joining method

IAA production was achieved when medium was supplemented with tryptophan (100 µg ml⁻¹). IAA production in presence of tryptophan (100µg ml⁻¹) in both the isolates with *A. chroococcum* CBD15 and *A. chroococcum* W5 producing 16.9 µg ml⁻¹ and 16.6 µg ml⁻¹ of IAA respectively (Fig. 5). There is no distinct differences were observed in IAA production in presence of tryptophan. However, in medium lacking tryptophan, IAA production drastically reduced in *A. chroococcum* CBD15 (4.4 µg ml⁻¹) and *A. chroococcum* W5 (4.2 µg ml⁻¹).

DISCUSSION

Several factors as excess ammonia, oxygen and temperature as well as other factors of the soil properties are reported to adversely affect the process of nitrogen fixation carried out by the soil microbial communities. The expression of *nif* genes for biological nitrogen fixation required functional nitrogenase. In our study, we aimed to

characterize two isolates of *A. chroococcum* (*A. chroococcum* CBD15 and *A. chroococcum* W5) with respect to nitrogen fixing ability and plant growth promoting (PGP) hormone production. Therefore, the sequence analysis of the gene *nifL* was carried out in both the *A. chroococcum* isolates under study so as to understand the molecular organization of the *nifL* gene in *A. chroococcum* and to establish its relationship with the already well studied *nifL* gene from *A. vinelandii*.

Phylogenetic analyses of *nifL* sequence revealed that isolates of *A. chroococcum*, CBD15 and W5 shared maximum homology (98%) with *A. vinelandii* with and *Pseudomonas oryzae* and are thus closely related to them. BLAST database to determine the unknown sequence data by comparing query sequences which already reported greater number of sequences in public domain at EMBL, NCBI and DDBJ (Andrew, 2000). The most conserved region of negative regulatory element in dizotrophic bacteria and

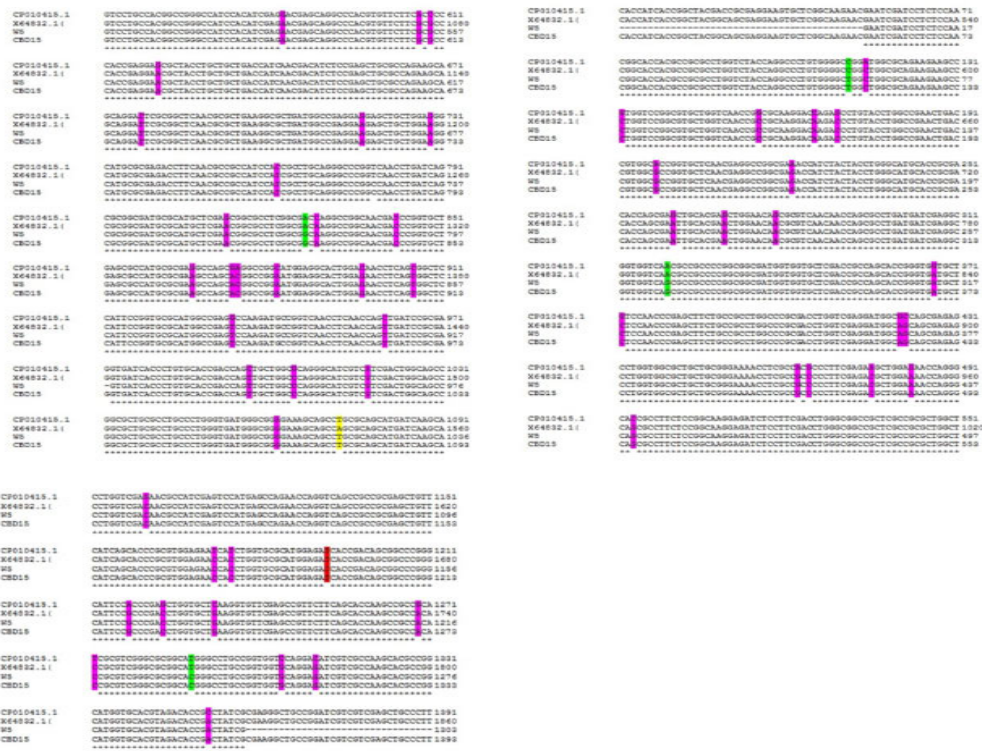


Fig. 2a. Homologies between the *nifL* gene of *A. chroococcum* of CBD15 and W5 and other negative regulatory gene from *A. vinilandii* and *A. chroococcum*. Homology to *A. chroococcum* of CBD15 and W5 *nifL* is shown highly identical with *A. vinilandii* (pink), *A. chroococcum* of CBD15 and W5 differ from other *A. vinilandii* and *A. chroococcum* (green)

worldwide accepted for phylogenetic analysis and classification of bacterium, to serving as molecular chronometer. Similarly, reported that partial *nifH* gene sequences provided the information on the classification of phylogeny of diazotroph natural communities (Franck *et al.*, 2001). Therefore, molecular characterization of genes is important get reliable and accurate identification of bacterial strains. The role of gene *nifL* in negative regulation of *nif* operon and ultimately nitrogen fixation pathway is well defined (Dixon, 1984; , Poza-Carrion *et al.*, 2014) The identification of closely related species was performed BLAST search. The bootstrap values ranged from 60- 100% which indicates *Azotobacteraceae* family (denoted at the node). *Azotobacter* was the first genus reported that having alternative nitrogenases: *vnfH* and *anfH* (Jianyini *et al.*, 2012). A segment of the *bat* gene in *Halobacterium halobium*, showed good

homology (30% identity) to residues 29-158 of *A. vinelandii nifL* gene product, and 29% identity *nifL* gene product of *K. pneumoniae* (Blanco *et al.*, 1993). The NifL protein regulate the expression of nitrogen fixation (*nif*) genes in presence of both the oxygen and fixed nitrogen or available nitrogen sources status. The negative regulator protein (NifL) decreased the expression of NifA, which transcriptional activator belonging to the bacterial enhancer-binding protein family. NifA is the gene product of *nifA* which is known to be positive regulator of *nif* operon (Martinez-Argudo *et al.*, 2004).

Nitrogen is a most essential element for crop improvement and yield through plant growth and development and forms an integral part of life cycle of all living forms on earth. The diazotrophic microorganisms through enzymatic nitrogen fixation play an important function in conversion of atmospheric nitrogen to soluble nitrogen in a form that becomes naturally available to plants and microbes in soil. The ability of converting acetylene to ethylene is an indirect measurement of nitrogenase enzyme, and is indicative of nitrogen fixing soil microbes (Andrade *et al.*, 1997; Manoj and Rajesh, 2015). The nitrogenase activity of isolates of *A. chroococcum* in our study was observed to decrease when nitrogen source was available in the media. Nitrogenase activity in *A. chroococcum* CBD15 when grown in a nitrogen source devoid medium decreased from 693.3 nmole C₂H₄ h⁻¹ mg⁻¹ to 365.0 nmole C₂H₄ h⁻¹ mg⁻¹ and

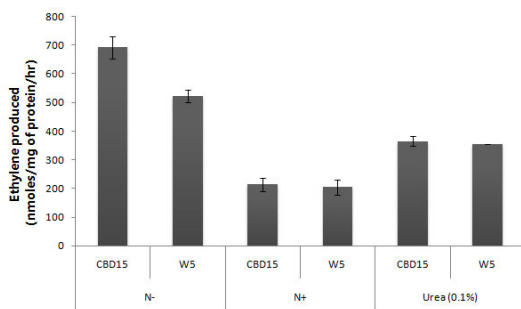


Fig. 3. Nitrogenase activity of *A. chroococcum* CBD15 and *A. chroococcum* W5 with different sources of nitrogen medium. Error bars refer to standard deviation by means of five replicates

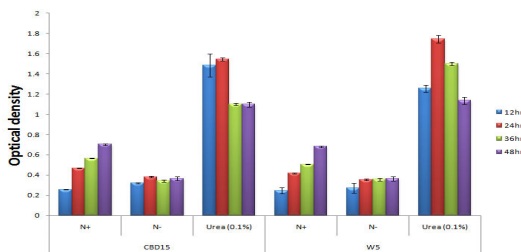


Fig. 4. Growth curve of *A. chroococcum* CBD15 and *A. chroococcum* W5 with different sources of nitrogen supplemented with medium. Error bars refer to standard deviation by means of five replicates

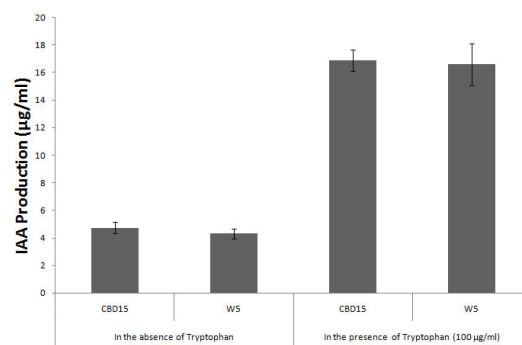


Fig. 5. Production of indole acetic acid (IAA) by *A. chroococcum* CBD15 and *A. chroococcum* W5 with presence and absence of tryptophan as precursor. Error bars refer to standard deviation by means of five replicates

213.2 nmole C₂H₄ h⁻¹ mg⁻¹ when grown in medium with 0.1% urea and medium with 0.11% of ammonium acetate respectively. Similarly in case of *A. chroococcum* W5 nitrogenase activity was found to be higher in media without nitrogen source (523.4 nmole C₂H₄ h⁻¹ mg⁻¹) in comparison to media with 0.1% urea (354.9 nmole C₂H₄ h⁻¹ mg⁻¹) and with 0.11% ammonia (204.4 nmole C₂H₄ h⁻¹ mg⁻¹) (Fig.3). (Fig.3). Previous reports also confirm that nitrogenase activity of nitrogen fixing bacteria rapidly decreased as ammonium concentration increased from 1mM (with glutamine concentration constant at 1.3 mM) in media to 10mM, and was finally switched off at ammonium concentration of 0.1 mM. At 0.1 mM ammonium, the rapid and total loss of activity was reversible and a 'switch-on' was observed in *Pseudomonas stutzeri* (Desnoves *et al.*, 2003). *A. vinelandii* is also reported that expression of all *nif* genes was reduced in the presence of external source of nitrogen. The negative regulatory gene, *nifL* is present immediately upstream of gene *nifA* which is considered to be positive regulator of the cascade of *nif* genes (Little *et al.*, 2012). Gene *nifA* was active in the absence of fixed nitrogen in the culture medium. Oliveira *et al.*, (2012) report that inactivation of NifA in response to fixed nitrogen, which transcriptionally regulated by δ^{54} transcriptional activator of *nifA* in *Herbaspirillum seropedicae*. The inhibitor NifL regulates NifA activity depending on the reduction status of its N-terminally bound FAD-cofactor and allows NifA expression under anaerobic conditions (Klopprogge *et al.*, 2002; Grabbe and Schmitz, 2003). However, *nif* gene expression is tightly controlled at the transcriptional level with respect to the fixed nitrogen concentration, external nitrogen source. The earlier studies in which *nif* cluster of *Paenibacillus* is subject to similar regulation in presence of ammonia and oxygen, *Paenibacillus* sp. WLY78 and the engineered *E. coli* 78-7 strain did not exhibit nitrogenase activity at oxygen and ammonia concentrations above 5 percent and above 1 mM respectively (Wang *et al.*, 2013).

Under natural conditions, bacterial isolates have the ability to produce phytohormones and successfully inhibiting pathogens by production of antibiotics, siderophores. Indole-3-acetic acid (IAA) involves cell enlargement and division, tissue differentiation, and responses to light

(Leveau and Lindow, 2005). The present results are similar with other studies where *Pseudomonas putida* has been shown to produce IAA when grown in a media supplemented with tryptophan as precursor of IAA. However, in medium lacking tryptophan, IAA production drastically reduced in *A. chroococcum* CBD15 (4.4 μ g ml⁻¹) and *A. chroococcum* W5 (4.2 μ g ml⁻¹). Similarly our results were agreed with a number of bacteria with plant growth promotion property such as *Azospirillum* sp., *Enterobacter cloacae*, *Alcaligenes faecalis*, *Acetobacter diazotrophicus*, *Klebsiella* sp., *Rhizobium*, *Klebsiellaoxytoca* and *Pseudomonas putida* (Leveau and Lindow, 2005). The minimum inhibitory effect of inorganic nitrogen on growth of *A. chroococcum* CBD15 was evident as the growth performance of the isolates *A. chroococcum* CBD15 and *A. chroococcum* W5 improved as evident from OD₆₀₀ which increased from 0.23 to 0.66 in 48 hours in presence of nitrogen sources (0.11% ammonium) in the medium.. However, no growth changes were observed in both the isolates when cultured in medium devoid of any nitrogen source, even after 48 hours. It was observed that there was rapid increase in growth of *A. chroococcum* CBD15 and *A. chroococcum* W5 within 24 hours when grown in media supplemented within 0.1% urea in the media as evident from OD at 600nm (Fig. 4). Mannitol used as carbon sources and isolated *Azotobacter* from plants rhizosphere (Shrivastava, 2013). Besides nitrogen source, micronutrients are also reported to influence the growth of *A. chroococcum*. Lu *et al.*, (2010) reported that *A. chroococcum* strains able to grow on a zinc-free medium as well as media containing a zinc concentration upto 6-40 ppm, They suggested that zinc concentration of 20ppm in soil was optimum for *Azotobacter* growth. Further studies in this direction with our isolates would prove useful in developing bioinoculants for biological nitrogen supply to crops.

In summary our results demonstrate that a negative regulatory gene (*nifL*) from an *Azotobacter* can inhibit the nitrogenase activity when trace amount of external nitrogen sources or available nitrogen. Also several reports were demonstrated deletion of *nifL* gene can overcome *nif* gene expression. It rises various questions which concerning the mutation for negative

regulatory elements (*nifL*) for fixing nitrogen efficiently by engineering diazotrophic eukaryotes through important biotechnological approach.

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