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Identification of Nitrogen Fixing Cyanobacteria By PCR Amplification of *Nif* Genes

KEYWORDS: Cyanobacteria, nitrogen fixation, nif gene, Cyanobacteria primers, Bio- fertiliser

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ABSTRACT *Nitrogen fixation is a common phenomenon exhibited by many organisms to fix environmental source of Nitrogen into simple utilisable form. Among various such organisms, Cyanobacteria possess nif genes, which can be identified at molecular level by Polymerase Chain Reaction. Cyanobacteria can be used as a potential Bio-Fertiliser for Kharif crops such as rice, maize, soybean, groundnuts, etc. The current study focuses on identifying nif genes containing Cyanobacteria from Ranchi, Jharkhand. The conserved sequences present on nif genes provide sufficient sequence information to construct primers, which were used to amplify nif genes.*

INTRODUCTION

Cyanobacteria, commonly known as the "Blue Green Algae" is the most commonly found, yet one of the most diverse group of organisms that have been existing in every possible terrain of the Earth since billions of years. Studies have concluded that it was Cyanobacteria that transformed the "Primitive Earth's" anaerobic environment to aerobic by converting the Oxygen in compound form to molecular form. Some of the species are capable to fix atmospheric Nitrogen (N_2) into the soil in the form of Ammonia (NH_3). This capability of Cyanobacteria has greatly astonished the scientific society, as the O_2 evolving and the N_2 fixing abilities does not generally co-exist.

In filamentous Cyanobacteria, for example, Anabaena, some cells differentiate into heterocysts when the cells are deprived of dissolved inorganic nitrogen. A heterocyst consists of a thick cell wall and only contains Photosystem I for ATP production. Photosystem II is degraded to prevent O_2 production. O_2 inhibits Nitrogenase, the enzyme responsible for N_2-fixation.

Nitrogenase consists of two proteins, MoFe Protein and Fe Protein. In a few other diazotrophs, the Fe protein of Nitrogenase undergoes reversible covalent modification (attachment of ADP-Ribose), rendering the Fe-protein inactive. Covalent modification (inactivation) of Nitrogenase is stimulated by ammonium or sources, by transfer of culture to dark or exposure to O_2. (Anonymous 2002)

Nitrogen is abundant in soil and atmosphere but in inorganic form (N_2).Crop plants such as pea, groundnuts, rice, wheat, sugarcane etc. cannot utilise this form of nitrogen since they do not possess the Nitrogenase enzyme, to convert it into a form that they can used to make proteins. Microbes, which include Cyanobacteria performs this work. They perform biological nitrogen fixation in which atmospheric nitrogen gas (N_2) is converted into ammonia (NH_3), that plants are able to use to synthesise proteins.

Among other Nitrogen fixing organisms, Cyanobacteria occupy a unique position in being the only true photoautotrophic aerobic Nitrogen fixing organism and are of great potential as Nitrogen-bio-fertiliser in Nature.

Cyanobacteria are one of the major members of "Microbial World" that provide potential source of Nitrogen fixation at nearly no cost. The bio fertiliser based on Cyanobacteria has been found to be highly supportive for the growth of crops or plants (Deepak *et al*, 2014).

The study was conducted to identify the potential N_2 fixing species of Cyanobacteria available in Ranchi city. The genomic DNA of each purified cyanobacterial strain was subjected to PCR based amplification of the gene sequence of *nif* S gene.

Recently, Assam became the first state of Bharat to be declared as an "Organic State". This can be achieved by other states also if instead of chemical fertilizers, use of Cyanobacteria based Bio-Fertilizers is encouraged.

MATERIALS AND METHODS

Sample collection

Cyanobacteria samples were collected from Dhurwa Dam, Ranchi Lake and a pond in Lalpur in Ranchi city, Jharkhand, in the months of November and December 2015, at regular interval of time (Table 1). The samples were inoculated in BG12 media and allowed to grow in liquid as well as on solid media. The culture was incubated at 23°C. Regular microscopic observations were made to ensure proper growth. The strains were isolated by the serial dilution method.

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Table 1: Sample Collection Details

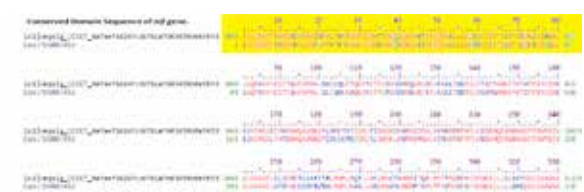
Sl No.	Site of collection.	Cyanobacterial Species Identified and Isolated.	Geographical Location of source.
1	Dhurwa Dam, Ranchi	Oscillator Pseudoanabaena Trebouxia Trebouxiophyceae	23.2937-85.259
2	Lalpur, Ranchi	Tolyphrix Pseudoanabaena Oscillatoria Nostoc Chroococcus turgidus	85.33774-23.37569
3	Ranchi Lake(Bada Talab)	Anabaena	23.3668-85.318

DNA ISOLATION

DNA was isolated from collected Cyanobacteria samples using CTAB method (Deepak *et al,* 2014).

PRIMER SYNTHESIS

Primers were synthesised using the NCBI Primer Designing Tool. Highlighted region of the conserved domain sequence (Figure 1) as shown in the photograph below, were used to design the primers.



PCR

Amplification was performed in a 20 µL reaction volume, consisting of 9µL nuclease free water, 1µL Taq polymerase, 10 pM concentration of forward and reverse primers and 30ng template. PCR was performed using a BioEra PCR machine. The thermal cycle was programmed for 2 minutes at 94 °C for initial denaturation, followed by 35 cycles of 30 seconds at 94 °C for denaturation, 30 seconds at 54°C for annealing, 40 seconds at 72**°C** for extension, and 5 min at 72°C for the final extension. PCR products were examined by Agarose Gel Electrophoresis at 100 volts for 30 min in a 1% (w/v) agarose gel in 1 x TAE buffer. PCR amplification of G3PDH gene in plant DNA that gives an amplification of 200bp was used as a marker due to the expected amplification is approximate 190bp from test samples.

RESULTS AND DISCUSSIONS

Sample isolation: samples collected were grown under provided condition as mentioned above. Single isolates were grown in liquid as well as solid BG 12 media {Figure 2(a) and 2(b)}. Regular microscopic observation confirms the single type of cyanobacteria isolated {Figure 3(a) and 3(b)}. Genomic DNA was isolated from these isolated cyanobacteria (Figure 4). Estimated 30 ng of genomic DNA used for PCR amplification of nif gene sequence. A G3PDH which is a gel marker for 200 bp amplification (primer are designed to amplify 200bp DNA fragment). 190bp amplification of *nif* genes from isolated cyanobacteria DNA was obtained which indicate the presence of nif genes in obtained isolates (Figure 5). Active expression of these nif genes for nitrogen fixation is possible from these strains, which need to be confirmed by expression profile study.

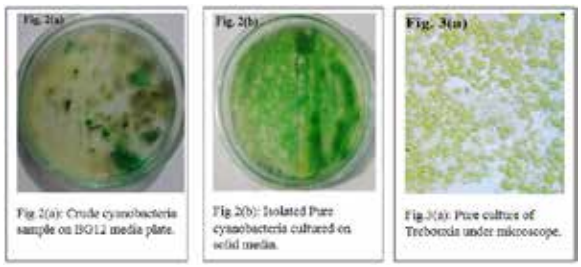


Fig 2(a): Crude cyanobacteria sample on BG12 media plate.

Fig 2(b): Isolated Pure cyanobacteria cultured on solid media.

Fig.3(a): Pure culture of Trebouxia under microscope.

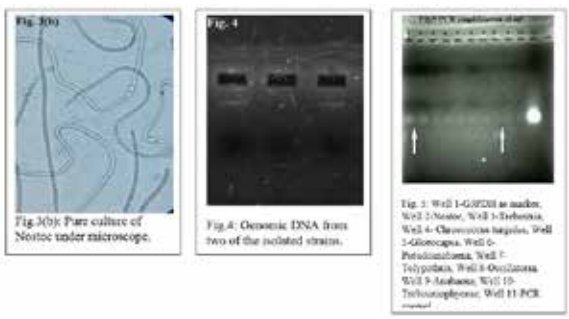


Fig.3(b): Pure culture of Nostoc under microscope.

Fig.4: Genomic DNA from two of the isolated strains.

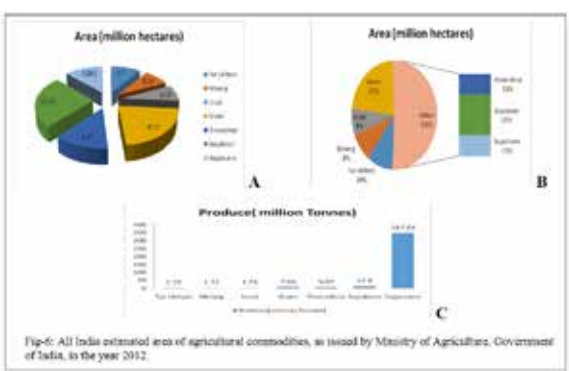


Fig-6: All India estimated area of agricultural commodities, as issued by Ministry of Agriculture, Government of India, in the year 2012.

The crops shared 19.08% of total agricultural produce on 4.75% of agricultural land in the year 2010-2011.

An independent study conducted in 2007-2008 ranked India last, among major countries applying organic farming in their fields. India uses just 0.3% of her agricultural land for organic farming. A major part of agriculture depended population in India still uses chemical fertilizers in the fields. This makes the soil lose its fertility in long span of time, making the farmers use more of the chemical fertilizers. This makes the farming costlier. The identified Cyanobacteria for N_2 fixation can prove to be an excellent alternative against the chemical fertilizers. Farmers can save more money by using a soil and environment friendly fertilizer. A cyanobacteria based fertilizer does not deteriorate, instead, adds up to the nutritional value of the soil.

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