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Isolation and Characterization of Nitrogen Fixing Bacteria from Agricultural Rhizosphere

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Abstract: *Present investigation on "Isolation And Characterization of Nitrogen Fixing Bacteria from agricultural rhizospere "was carried out with the objectives of Isolation and Characterization of Nitrogen Fixing Bacteria. A study was undertaken to investigate the occurrence of Nitrogen Fixing Bacteria from Soil and Root nodule of Nashik area. Four soil samples and two root nodule samples were collected randomly to estimate microbial population which used plate count method. The present study describes the characterization of Nitrogen Fixing Bacteria strain isolated from leguminous plant species.\The characterization of isolated pure cultures through colony morphology analysis, cellular morphology and biochemical properties are including gram staining, catalase test, Methyl red test, voges proskaeur test and citrate utilization and nitrate reduction pattern Isolation of DNA was done with the modified CTAB method .The isolated DNA was subjected to Agarose gel electrophoresis and observed under UV light. The presence of nifH check by using PCR which gave 700bps amplicon in 03 isolates. These isolates will be useful to produce efficient biofertilizers for Agriculture.*

Keywords: *NFB, nifH gene, morphological, Biochemical test, amplicon.*

I. Introduction

Nutrient enrichment of soils by nitrogen fixing symbiotic bacteria present in legumes has been known for centuries. Rhizobium spp. are well known group of bacteria that acts as the primary symbiotic fixer of nitrogen. These bacteria infect the roots of leguminous plants, leading to the formation of lumps or nodules where the nitrogen fixation takes place. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium. This symbiosis reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops [1]. The isolates were grown on yeast extract – mannitol agar (YMA), and the morphologic characterization was realised by Gram stain [4]. Rhizobium is the root of legumes host nitrogen fixing bacteria which can invade root and get sugars from the plant. In return, they convert large amounts of dinitrogen (N2) from the atmosphere into forms that the plants can use [3]. We use specific primer for Rhizobia to recognize the genetic variation between the isolates which is deferens in their efficiency of nitrogen fixation and tolerance of biopesticide.

II. Material And Methods

2.1 Collection Of Sample

Collection of soybean soil and root nodule from the nursery ,then collection of chick pea soil and root nodule from college farm (KKWCABT, Nashik) and collection fava bean and black gram soil from the farm of nandur naka area district Nashik (India). It was kept in refrigerator at 4^0C.

2.2 Isolation Of Nitrogen Fixing Bacteria:

The Nitrogen Fixing Bacteria was isolated from the soil , chick pea & soybean root nodule. The active nodule was distinguished according to it colored (pink) and size (large) [2]. The bacteria isolated from agricultural soil & root nodule at the dilution of 10^{-7} to 10^{-9} on YEMA-CR media for 48 hrs at 32^{0C}. Bacterial culture were repeated for three times for better pure culture isolation of bacteria. Identification of the isolates were done by morphological & various biochemical methods.

2.3 Morphological Characterization

Isolates were characterized by cell (Gram stain, determination of the presence of spores), colony (shape, colour**,** margin, Nature of Colony, texture,) was observed.

2.4 Biochemical Characterization

Isolates were characterized by different biochemical methods nitrate Reductase test, citrate utilization test, methyl red test, VP test, Catalase test (cover slip method), and Oxidase test.

2.5 Molecular Characterization:

2.5.1 Genomic Dna Isolation & Purification:

Isolated bacteria were cultured in LB medium on a shaking incubator at 30˚C for 18 hours. These cultures were then centrifuged at 12000 rpm for 2 minutes. Genomic DNA was extracted using C-TAB method given by [13].

2.5.2 Amplification of nifH gene:

A 25-µL PCR reaction mix was prepared using primers: 5′TTCCATCAGCAGCTCTTCGA3′ and 5′GGCAAAGGTGGTATCGGTAA3′(Eurofins Banglore). The PCR thermo cycling condition was 95˚C for 7 minutes (preheating), 95˚C for 45 seconds, 54˚C for 1minutes, and 72˚C for 45 seconds and 35 cycles, followed by a final heating at 72˚C for 10 minutes. The PCR product size was confirmed by electrophoresis on a 1% agarose gel (gel documetation system UV-TECH, Banglore). All reagents, Taq polymerase and DNA ladder were purchased from Himedia [10].

III. Results

3.1 Isolation of NFB

A total six isolates were isolated were isolated & identified from agricultural rhizospere & root nodules among them 05 strains shown properties of Rhizobium spp. And 01 strain of Azetobactor spp. Isolation done on YEMA-CR media and pure culture maintain on NA media (Fig: 1)

3.2 Morphological & Biochemical Characterization

Isolates were characterized by cell (Gram stain, determination of the presence of spores), colony (shape, colour**,** margin, Nature of Colony, texture,) was observed (Table: 1) . various biochemical test performed on isolates for characterization according to Burgys Manual. Some isolates shown positive results towards the biochemical test (Table: 2 & Fig: 2)

3.3 The Presence of nifH Gene

The total genomic DNA isolation was done by C-Tab method of Sambrrok & Ruseel. He DNA isolation shown distinct high molecular weight band in all 06 isolates without any contamination (fig: 3). Molecular characterization of 06 NFB isolates was carried out in order tocheck the presence of nifH gene. A method given by Nemat et.al 2013 for nifH gene primer was used for amplifying nifH gene gave uniform distinct amplicon of 700bp. Out of 06 isolates three isolates i. e. R3, R4 & R5 gave distinct dark band of 700bps & R1 & R2 shown less dense band at 700bp. No amplification of nifH gene obtained in R6 isolate (fig: 4)

IV. Discussions

Present investigation was carried out at the Department of Biochemistry and Molecular Biology, K. K. Wagh College of Agricultural Biotechnology, Nashik, during the period from December 2014 to Aril 2015, with the objectives of Isolation and Characterization of Nitrogen Fixing Bacteria**.** Many various strains were found during the isolation and characterization of bacteria originating from the nodules of several leguminous plants and from the soil of the rhizospere. These strains probably represent 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 first time in a natural mountainous habitat of Rhizobia associated with leguminous plants which were not cultivated. 5 Isolates showing the characterization properties of rhizobium and one showing properties like azetobactor [7].

The bacteria isolated from rhizospere soil & nodules by serial dilution 10^{-7} to 10^{-9} on YEMA-CR medium [1]. isolates characterized by morphologically & biochemically according to burgis manual. Which shown features of Rhizobium spp. Genomic DNA isolated by C-TAB method which was modified as for isolates by increasing concentration of C-TAB & SDS [13]. The nifH gene primer used for amplification which gave better amplicaon at 54^{0c}. The 03 isolates i.e. R3, R4 & R5 shown distinct band at 700bp. Where there was no presence of nifH gene in R6 isolate. The same way results obtained by Neamt et al., [10].

It was found that these Nitrogen Fixing strains along can substitute the chemical fertilizer, might be used to reduce the alkalinity of soil by neutralization phenomenon through organic acid exudation and can survive in the soil system to retain the Nitrogen fixing potential for long time.

V. Tables & Figures

Table: 1 - Morphological Characterization of Bacterial isolates

Isolates	Colony shape	Cell Shape	Colony colour	Transparency	Nature of Colony	Margin of Colony	Surface of colony
Soybean Soil (R1)	Circular	Rod	White	Unopaque	Unglistening	Entire	Smooth
Soybean Root Nodule (R2)	Circular	Rod	White	Opaque	Glistening	Entire	Smooth
Chick Pea Soil (R3)	Circular	Rod	creamy	Unopaque	Unglistening	Entire	Smooth
Chick Pea Root Nodule (R4)	Circular	Rod	creamy	Opaque	Glistening	Entire	Smooth
Fava Bean Soil (R5)	Circular	Rod	creamy	Opaque	Glistening	Entire	Smooth
Black gram Soil (R6)	Spherical Shape	Rod	White	Opaque	Glistening	Entire	Smooth

Table: 2 - Biochemical Characterization of Bacterial isolates

Test name	Soybean soil R1	Soybean root nodule R2	Chick pea soil R3	Chick pea root nodule R4	Fava bean soil R5	Black gram soil R6
Gram staining	+(Gram negative)	+(Gram negative)	+(Gram negative)	+(Gram negative)	+(Gram negative)	+(Gram negative)
Citrate utilization test	+	–	+	+	+	+
Methylred test	+	+	+	+	+	+
Voges prosqure test	–	–	–	–	–	–
Catalase test	+	+	+	+	+	+
Nitrate Reductase Test	+	–	+	+	+	+

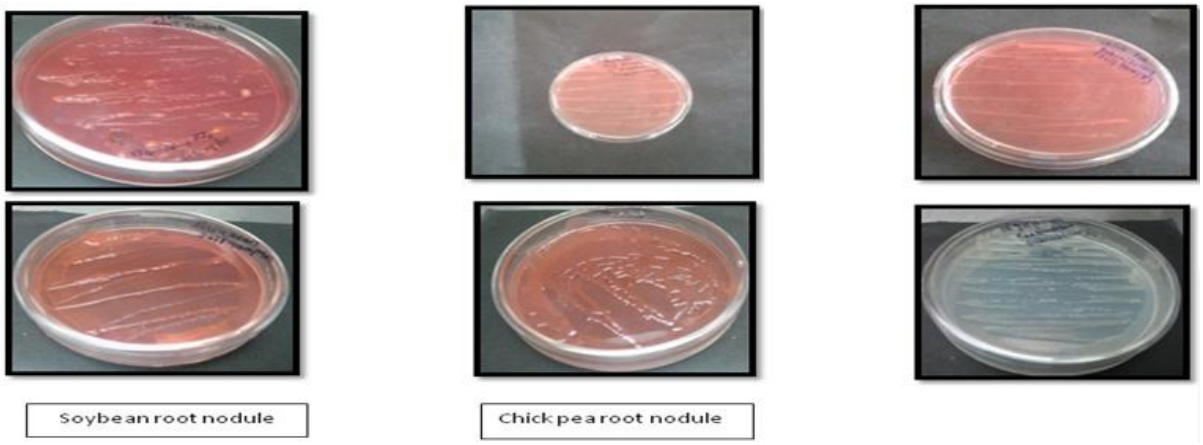
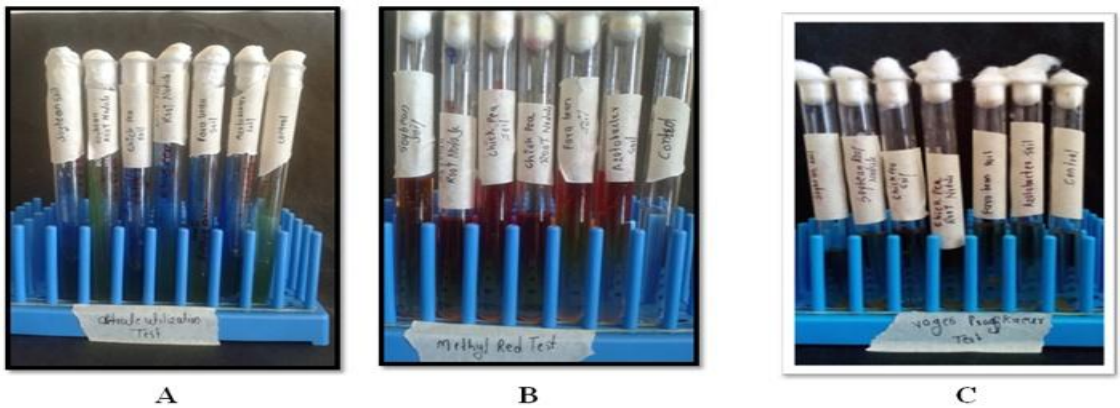


Fig: 1 Bacterial strains isolated Agricultural soil & root nodules.



A B C

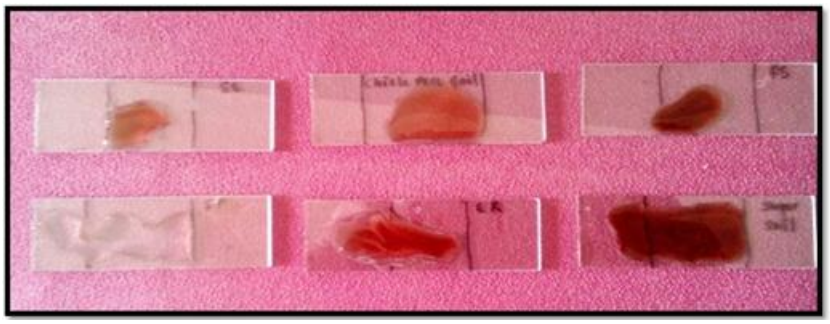


Fig: 2 Biochemical characterizations of bacterial isolates.

(A- Citrate utilization test, B- methyl red test, C- Vogues proskur test & D- Catalase test)

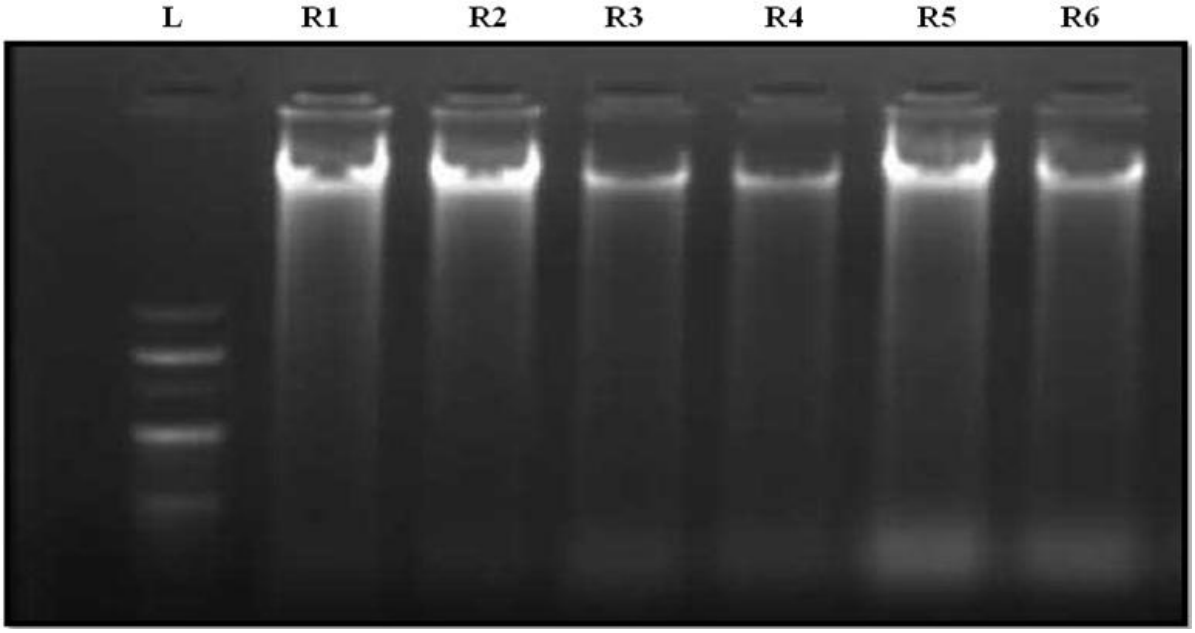


Fig: 3 Isolated genomic DNA from isolates by C-TAB method

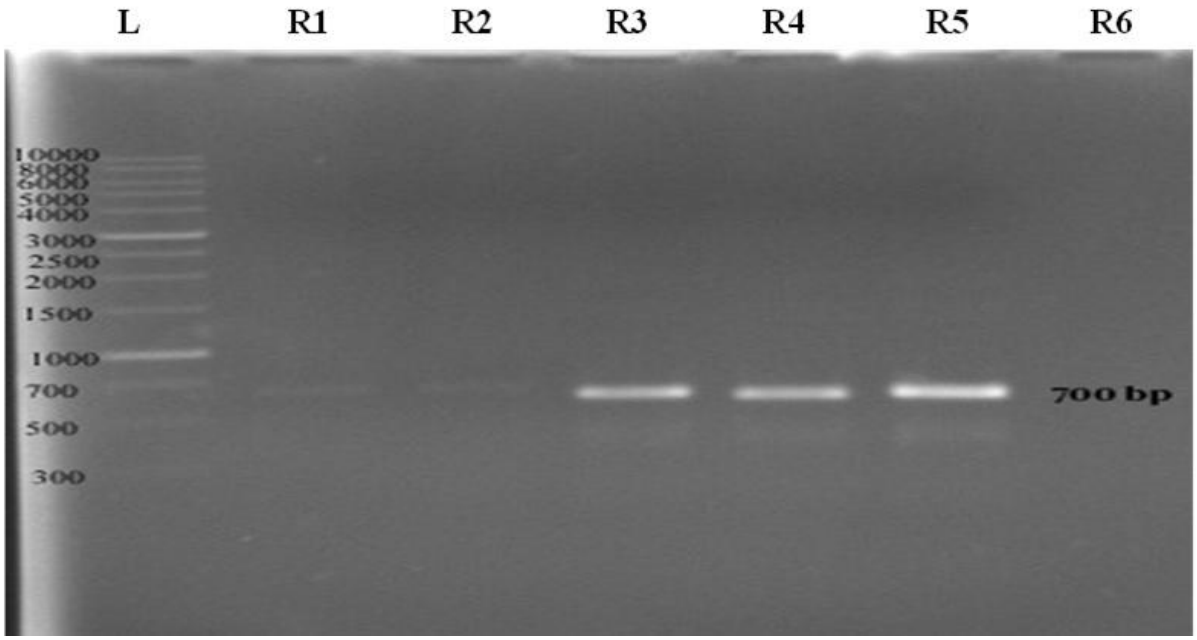


Fig: 4 Amplification for the presence of NIFH gene in local isolates

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