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Study of Phytobeneficial Characterization and Biofertilizer Efficacy of *Rhizobium* Strain Isolated from Pulse Fields of Odisha

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Haphazard and indiscriminate application of chemical fertilizers has led to environmental contamination including deterioration of soil health. Hence it is very essential to inculcate biological agents in order to reduce the harsh effects of chemical fertilizer. The use of potent bacteria as a bio-fertilizer can improve the soil nitrogen status leading to enhanced soil health and improved productivity. *Rhizobium* provides a major source of nitrogen input in agricultural soils. On account of that, 64 bacteria were isolated and subjected to RT-PCR for nifH gene quantification to ascertain biological nitrification. From these 64, Seventeen potent PGP bacteria, isolated from different pulse fields, showing maximum PGP traits were studied further for nif H quantification. Out of 17, five were found to be possessing highest number of copy numbers of nifH gene indicating efficient nitrogenase activity. On molecular identification, it was revealed that except BS5JGS, rest all were belonging to the Enterobacteriaceae family. BS5JGS was found out to be *Rhizobium* sp., with an accession number assigned as OK602696. An array of organic acid production by the *Rhizobium* strain was estimated by HPLC, in which adipic acid was detected in highest concentration followed by tartaric acid, malonic acid, citric acid, ascorbic acid and phosphoric acid. The Rhizobial isolate was found to inhibit two most common fungal phyto-pathogens *Fusarium oxysporum* (ITCC 4998) and *Rhizoctonia solani*(ITCC 2060). It showed various phytobeneficial traits which imply it can be utilized as an effective biofertilizer for legumes and pulses as well as to maintain soil fertility and soil nutrient status.

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1. INTRODUCTION

With the rampant increase in population coupled with decreasing cultivable land and climate change, there is an increasing demand for more production of food from within the available resources. The world's growing population will require a 70% increase in agricultural production by 2050 [1].

"The strategies developed by the Green Revolution focused on fending off starvation and was very successful in raising overall yields of cereal grains, but did not give sufficient relevance to nutritional quality. High-yield-cereal crops have low-quality, with essential amino acid deficiencies, are high in carbohydrates, and lack balanced essential fatty acids, vitamins, minerals and other quality factors" [2]. Over the period, the soil microbial population has drastically declined. In the present context of environmental deterioration, researchers are emphasizing on Green revolution II, where organic farming is prioritized and soil is amended with organic manures and biofertilizers to increase productivity. Emphasis is being given to reduce the application of chemical fertilizer or agrochemicals in agriculture.

"Odisha is known to grow and cultivate pulses with an annual production of 10.6 lakh tons, at the rate of 508 kg/h" [3]. "The major pulses grown in the state are green gram, black gram, pigeon pea etc. in symbiotic association with nitrogen fixing bacteria. The Nitrogen fixing efficacy of bacteria can be ascertained by determining the presence of nitrogenase enzyme" [4], where "the potential activity, structural and functional data can be linked to the molecular measurements of functional nif gene abundance" [5].

"The nif genes are the marker genes which encode primarily for the nitrogenase enzyme present in both free-living & symbiotic bacteria and can specify the effectiveness of the organism in the nitrogen fixation process" [6].

"In addition to nif H, plant growth-promoting traits can determine the potential of isolates in enhancing growth and crop yield" [7,8] "through various direct and indirect mechanisms viz.: potassium and phosphate solubilisation, nitrogen fixation, phytohormone & siderophore production, hydrolytic & antimicrobial enzymes biosynthesis, induced systemic resistance, antioxidative defence mechanisms etc" [8-13]. In view of this, the present study aims to evaluate the nitrogen-fixing efficiency and biofertilizer efficacy of Rhizobium strains isolated from the rhizospheric region of pulse crops from different pulse fields of Odisha.

2. MATERIALS AND METHODS

Collection of Samples:

Soil and root nodule samples from the rhizospheric region of green gram, black gram and pigeon pea were collected aseptically from different pulse fields of Odisha. The experiment was conducted during the year 2018-19, in the laboratory of Department of Microbiology, CBSH, OUAT, Bhubaneswar, Odisha, India.

Isolation and Screening of PGPR Traits:

Soil samples were serially diluted, cultured in Nutrient agar plates and incubated at 37°C for 48hrs. The root nodules were subjected to surface sterilization using 0.01% $HgCl_2$ for 2 minutes followed by continuous washing with sterile distilled water. The nodules were then crushed between two clean and sterile glass slides and the obtained exudate was streaked onto the CRYEMA medium plates. Only transparent to translucent, white, mucoid, slimy colonies were marked and selected for further study. A total of 64 bacteria were isolated and named as BS 1 to BS 64.

Confirmatory tests for *Rhizobium*:

The obtained bacterial isolates were checked for contamination and particularly to distinguish the *Rhizobium* from *Agrobacterium* using the following confirmatory biochemical tests:

CRYEMA medium:

YEMA medium incorporated with 2.5 ml of 1% aqueous solution of Congo red dye per 1 litre of YEMA medium results in white, translucent, elevated and glistening in morphology and are comparatively smaller with an entire margin in contrast to stained *Agrobacterium* colonies [14]. According to Fred *et al*. (1931), the media consists of Mannitol (10g), K_2HPO_4 (0.5g), $MgSO_4.7H_2O$ (0.2 g), NaCl (0.1g), Yeast Extract

(1g), $CaCO_3$ (1g), Agar–Agar (20g), and distilled water (1000ml) with a final pH of 7.0. Sterilization of the media was achieved by autoclaving at 15 PSI at 121^{0}C for 15-20 min. Upon incubation of 48-72 hrs, the morphologically desired colonies are selected for further study. *Rhizobium* usually does not take up the dye and is whitish in colour whereas *Agrobacterium* colonies are pink to red in colour.

Bromothymol blue test:

CRYEMA medium amended with 0.001% Bromothymol blue as an indicator. If there is acid production the medium turns yellow as in the case of fast-growing *Rhizobium* sp.

Glucose peptone agar test:

Rhizobia lacks the ability to utilize peptone unlike *Agrobacterium* which shows a fast and luxuriant growth on glucose peptone agar. The media composition is as follows-Glucose-10g, Peptone 20g, NaCl-5 g, Agar–Agar- 20 g, distilled water 1000 ml with a maintained pH 7. 2 also amended with 1 mL of 1.6% Bromocresol purple per litre of medium. Post incubation of 3-4 days, the colonies are differentiated from the Agrobacterium contamination by their growth characteristics. Bacterial isolates exhibiting poor to no growth with neutral or alkaline reaction were further selected as Agrobacterium is acknowledged on the basis of acidic reaction and maximum growth on the medium. Colour change of the medium to yellow indicated acid production by Agrobacteria and other contaminants [15].

Keto-lactose medium test:

Keto-lactose medium was prepared by splicing mannitol with lactose in YEMA medium. The medium was sterilized by steaming for a duration of 30 min. After sterilization the medium was allowed to cool at room temperature (30±2^{0}C) and stored at the same temperature for 2 to 3 days to check for contamination and corroborate its sterility. A loopful of the inoculum from a fully grown 7 days old culture slant was transferred to the prepared keto-lactose agar medium. Post incubation of 5-7 days at 27-30^{0}C, the plates were flooded with Benedict's solution.

Composition of benedict's solution:

- Solution A (Sodium citrate-173 g; Anhydrous sodium carbonate-100 g; Distilled water-600 ml)
- Solution B (Copper sulphate-17.3 g; Distilled water-100 ml)

Both these solution A and B were separately prepared. Later, solution B was mixed with solution A and subjected to filtration. The resulting clear and transparent blue solution was poured onto the medium plates consisting the bacterial cultures. Excess solution was decanted and the plates were incubated for one hour without any disturbance at room temperature 30±1^{0}C. the presence of *Agrobacterium* is confirmed by the yellow coloration of medium around the bacterial colonies [16].

Hofer's Alkaline Medium Test:

Agrobacterium has the ability to grow at higher pH levels unlike Rhizobium. Therefore, their growth in Yeast extract mannitol broth with elevated pH of 11.0 can help with distinguishing between the two allied genera [17].

Reduction of 2, 3, 5 Triphenyl Tetrazolium Chloride (TTC) Herrigan et al. (1966):

TTC is considered to be a redox indicator which indicates the capability of the isolates for dehydrogenase enzyme production. The bacterial isolates were inoculated into the tubes containing 5 mL of YEM broth and kept for incubation at 28^{0}C for 7 days. After incubation, 1% solution of 2, 3, 5 TTC was added and further incubated for 30 min at 28^{0}C. Pink colour appearance in the tubes indicated the reduction of TTC.

NaCl Tolerance:

Growth of the organisms on YEMA medium supplemented with 1 to 8% NaCl was checked. Highly diluted suspensions of the organisms were spot inoculated on the plates, incubated at 30 ± 1ºC for 72 hrs and growth was recorded.

The obtained rhizobacterial isolates were screened further for nif H quantification. Organisms with maximum plant growth promoting traits were taken for the further study.

Extraction and Purification of DNA:

The 17 bacterial isolates were subjected to DNA extraction. The cultures were inoculated in sterile

luria bertani broth and incubated at 35 ± 2 °C for 72 hrs and DNA was extracted using the bacterial DNA extraction kit (Sigma-Aldrich-GDI3 MSDS), quantified through Nano-3100 Analytical Spectrophotometer and kept at -80°C for further analysis.

Quantification of nifH Gene Copy Number by Real-time PCR:

NifH gene copy numbers were quantified and measured in Real-time polymerase chain reaction (RT-PCR) (QuantStudio® 5, Singapore) by the addition of an intercalating fluorescent dye SYBR® Premix Ex Taq™ (Takara Bio Inc., Japan). Quantitative PCR (qPCR) protocol was carried out using universal primer sets (in 10 µM concentration) *nifH*-R (5′TTGTTSGCSGCRTACATSGCCATCAT3′) and*nifH*-F(5′AAAGGYGGWATCGGYAARTCCA CCAC3′) [18,19]

Quality of plasmid DNA was quantified and evaluated through Nano-3100 (Analytical®) spectrophotometer and sequenced thereafter. To evaluate and assess the copy number of *nifH* gene, plasmid DNA was diluted and a standard was prepared ranging from 3×10^1 to 3×10^8 copies. The nif H copies were calculated from a standard constructed by plotting plasmid DNA concentrations versus quantification cycles which produced linear $R^2 > 0.97$ standard curve.

Secondary Screening for PGP Traits:

Indole Acetic Acid (IAA) production: The test is used to check ability of the organisms to form indole from tryptophan or to detect the presence of enzyme tryptophanase which converts tryptophan to indole. The test was performed by inoculating the bacterial cultures into tubes containing tryptone broth incubated at 30 ± 1ºC for 72 hrs. After inoculation, Kovac's or Salkowski's reagent was added (1:1 by volume) and mixed to check for indole production which was indicated by a pink ring at the interface of the two solutions.

Solubilization of inorganic phosphate:

For phosphate solubilization assay, fresh cultures of bacterial strains were inoculated into National Botanical Research Institute Phosphate (NBRIP) Medium [20]. Plates were incubated for 7 days and the formation of clear zone around the colonies were observed. The solubility index of the bacterial isolate was calculated by the following formulae [21].

Solubility index = [halozone diameter (mm) – colony diameter (mm)] / colony diameter (mm)

Ammonia production:

Bacterial strains were checked for ammonia production according to Cappuccino and Sherman [22]. Fresh cultures were inoculated in to 10 ml peptone water. The production of ammonia was detected by adding 500 µl of Nessler's reagent to each tube.

Siderophore production:

Detection of Siderophore production was carried out by inoculating the bacterial isolates on Chrome Azurol S (CAS) plate assays [23]. This assay was carried out based on the competition for iron between the ferric complexes of an indicator dye, Chrome Azurol S (CAS) and the siderophores produced by bacteria which apparently have a higher affinity for chelating Fe^{+3} of CAS. Siderophore production by rhizobacterial isolates was detected as described by Schwyn and Neilands [23]. The assay was performed by using CAS agar medium which contains the ternary complex CAS/Fe^{+3}/hexadecetyltrimethyl-ammonium bromide as an indicator. Autoclaved CAS agar medium was poured in each Petri dish. The rhizobacterial inoculum was spot inoculated in the medium. The plates were incubated in the dark at 30ºC for 7 days.

HCN production:

The HCN production by the bacterial isolates was determined by following the method of Ahmad *et al.* [24]. Fresh cultures were inoculated in LB medium supplemented with glycin (4.4g/lit) for 24 h at 30^0C.On the top of each plate, a sterilized filter paper (Whatman no.1) soaked in 2% sodium carbonate prepared in 0.5% picric acid solution was placed. Plates were sealed and incubated at 30^0C for 4 days to study HCN production.

Zinc solubilization:

Zinc, being an imperative micronutrient are essentially required for optimum plant growth. Zinc solubilizing bacteria are potential alternatives for zinc supplementation and convert applied inorganic zinc to available forms.

The ability of the bacterial isolates to solubilize zinc was screened by growing them on HiMedia Zinc solubilizing agar medium plates. After an incubation period of 5days, the plates were checked for halozones around the colonies. The halozones indicate the solubilization of zinc by the bacterial isolate. The solubility index of the bacterial isolate was calculated by the following formulae [21].

Solubility index = [halozone diameter (mm) – colony diameter (mm)] / colony diameter (mm)

Potassium solubilization:

After nitrogen and phosphorus, potassium is a key element involved with the growth and metabolism of plants. The ability of the bacterial isolates to solubilize potassium was estimated by plate assay wherein, the bacterial cultures were inoculated on Alexandrow agar medium (HiMedia). After incubation of 5-7 days, the plates were checked for halozone around the colonies which indicated potassium solubilization.

The solubility index of the bacterial isolate was calculated by the following formulae [21].

Solubility index = [halozone diameter (mm) – colony diameter (mm)] / colony diameter (mm)

2.1 Amplification and Evolutionary Analysis of 16s r-DNA

The extracted DNA was sent to Eurofins Genomics India Pvt. Ltd. Bangalore, India, for 16s r-DNA sequencing. The quality of extracted DNA was evaluated on 1.2% gel using 1kb ladder.16s r-DNA fragments were amplified and a single discrete PCR amplicon band resolved on the agarose gel. The amplified product was then purified in order to remove any contaminants. Forward and reverse DNA sequencing reaction was carried out using BDT v3.1 Cycle sequencing kit on ABI 3730x1 Genetic Analyzer. Consensus of 16s r-DNA was analysed by Bio-Edit software v7.0.5.3. By using Clustal W v 1.6, the submitted 16s r-DNA sequence of the bacterial isolates were subjected to multiple sequence analysis. The phylogenetic tree was constructed using neighbor-joining method [25] of MEGA 6.06 (Molecular Evolutionary Genetics Analysis) software [26]. Evaluation of the resultant phylogenetic tree topologies were done by bootstrap analysis of neighbor-joining data sets.

2.2 Suppression of Fungal Phyto-pathogens (in-vitro) by Potent Rhizobium Isolate

Suppression of fungal phytopathogens (in-vitro) activity of potent bacterial isolate was studied by dual culture method [27]. For antagonistic activity study two different fungal phytopathogens such as *Fusarium oxysporium* ITCC 4998 and Rhizoctonia solani ITCC 2060 were selected. A loopful of 24 hours fresh culture of bacterial isolate was streaked as a straight line (3cm) away from the mycelial spot at two opposite edges of petri dishes (90 mm). The plates were incubated at 28°C for 5 days to observe their degree of inhibition. This test was conducted in triplicate with a control (without bacteria). The degree of fungal radial growth

$$\text{Inhibition (\%)} = \frac{(\text{Control} - \text{Treatment})}{\text{Control}} \text{x } 100$$

Determination of Organic Acid Produced by the Bacterium using HPLC:

For analysis of organic acids, the bacterial isolate was inoculated in a 250 ml conical flask containing 50 ml of NBRIP broth medium in triplicate. The flasks were incubated at 37°C temperature in an orbital shaker at 100 rpm for 144 hrs. One millilitre of incubated sample was centrifuged at 10,000 rpm (Mikro-200, Hettich Zentrifugen, Germany) for 15 min. and filtered through 0.22 µm nylon membranes (Pall India Pvt. Ltd.) to obtain cell-free culture supernatant. Twenty microlitres of filtered supernatant was injected to HPLC (LC- 10AT, Shimadzu). The organic acid separation was carried out on an ion exclusion column Aminexs$^{@}$ HPX-87H 300 mm X 7.8 mm (Bio- Rad Laboratories Inc.) with 0.008 M H_2SO_4 as mobile phase at a constant flow rate of 0.6 ml/min and at operating temperature of 30°C. The retention time of each test signal was recorded at a wavelength of 210 nm (SPD 10A, Shimadzu) and compared with organic acid analysis standard kit (Bio-Rad Laboratories Inc.) following the methods of Yadav et al. [28].

Quantification of Phosphate, Zinc and Potassium Solubilisation by the Bacterial Isolate using Inductively Coupled Plasma Optical Emission Spectroscopy ICP-OES:

For the quantification of Phosphate and Zinc solubilised by the isolate, 100mL of NBRIP medium amended with 0.1% Zinc carbonate was prepared and inoculated with the bacterial culture. It was then incubated at 28°C for around 6 days on a shaker incubator with 100rpm. Before inoculation of the culture, around 1mL of broth medium was pipetted out as 0-day sample. The broth was centrifuged at 6500 rpm for 15 mins and the supernatant was collected, which was then filtered through Millipore filter membrane and stored at 4°C for further analysis. Same procedure was repeated every alternate day to obtain the samples with regular intervals. Similarly, for the quantification of Potassium, 100 mL of Alexandrow's broth medium was prepared and inoculated. Same procedure was repeated as in the case of Phosphate and Zinc. All the samples were filtered and diluted 10X. The samples were then subjected to ICP-OES analysis for the estimation of the total amount of Phosphorus, Zinc and Potassium solubilised in the medium by the bacterial isolate throughout the incubation process of 6 days. The 0-day samples were taken as control samples wherein no bacterial culture was inoculated.

Evaluation of Effective PGPR *Rhizobium* Isolate on Seed Germination and Growth:

Roll towel method: The experiment was conducted with Green gram to assess the effect of the potential PGPR Rhizobium isolate on seed germination and plant growth promoting abilities by the standard roll towel method (ISTA, 1985) in germination paper. Locally purchased seeds of green gram were used for this experiment. The seeds were surface sterilized with 0.2% $HgCl_2$ solution for 2-3 min, and air dried for 15 minutes. The seeds were soaked in 10 ml of the bacterial suspension (10^9 cfu / ml) for 30 minutes. A control was run with sterile nutrient broth for comparison. Then the seeds were blot dried, placed in wet blotters and in germination paper maintained at 27 ± 2^0C. On the fifth day, the germination was recorded. Ten seedlings from each were taken for determination of root length and shoot length separately.

3. RESULTS AND DISCUSSION

The confirmatory tests revealed 17 out of the total 64 bacterial isolates were showing traits similar to that of *Rhizobium* sp. and thus, were selected further for the study.

NifH Quantification using RT-PCR:

Extraction and purification of DNA: Absolute Quantification of nifH gene copy number by Real-time PCR. Transcription of nifH gene is strictly regulated by the level of molecular oxygen and fixed N_2 to minimize unnecessary energy consumption [29] and is strongly related to the N_2 fixing ability of the plant [30]. Therefore, the detection of nifH amplified from mRNA indicates the presence of N_2-fixing bacteria as well as evidence of N_2 fixation in plants [31]. Out of the 17, 5 isolates BS2 JGS, BS5 JGS, BS25 BHU, BS29 ANG and BS45 GNJ showed maximum nifH copy numbers. By plotting with best three points of plasmid DNA concentrations *versus* critical threshold (*Ct* mean), the gene copy number (nifH) per mL culture was calculated from a linear standard curve (R^2 = 0.987), where slope m= -2.382. Maximum copy number of nif H gene was found in BS5 JGS.

The 5 bacterial isolates showing maximum nif H gene quantification, were selected for the plant growth promoting parameters.

Secondary Screening for PGP Traits:

The potent nitrogen fixing bacterial isolates were further screened for plant growth promoting traits, wherein it was found that, three out of the 5 isolates were siderophore producers, all of them were positive for ammonia and IAA production, three of them were found to be phosphate and zinc solubilizers, two of them were potassium solubilizers, all of them were nitrogen fixers and none of the isolates showed HCN production.

These results indicate that the tested isolates could exhibit more than two or three plant growth promoting (PGP) traits, which may promote plant growth directly, indirectly, or synergistically. Similar to our findings of multiple PGP activities among PGPR have been reported by some

Table 1. Confirmatory biochemical tests for *Rhizobium* sp. as shown by the 17 bacterial isolates

Isolates	CRYEMA medium	Bromothymol Blue medium	Glucose peptone agar	Keto-lactose medium	Hofer's Alkaline medium	TTC Reduction	Gram character
BS 2 JGS	Off-white mucoid	Yellowish green coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS 5 JGS	Off-white mucoid	Bluish colour of media	Poor to no growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS 7 JGS	Off-white mucoid	Bluish-green coloration	Poor to no growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS 16 PUR	Off-white mucoid	Yellowish green coloration	Poor to no growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS 17 PUR	White mucoid	Yellow coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS 19 ANG	Translucent mucoid	Bluish-green coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS 24 BHU	Off-white mucoid	Bluish coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS 25 BHU	Off-white mucoid	Bluish coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS 26 BHU	Off-white mucoid	Bluish green coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS 29 ANG	White mucoid	Bluish coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS37ANG	White mucoid	Bluish coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS45 GNJ	Off-white mucoid	Bluish green coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS46 GNJ	Off-white mucoid	Yellowish green coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS50 JGS	Off-white mucoid	Bluish coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS51 JGS	Off-white mucoid	Bluish coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS52 JGS	Off-white mucoid	Bluish coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli
BS 53 JGS	Off-white mucoid	Bluish coloration	Poor growth	No growth	No growth	Pink coloration	Gram negative bacilli

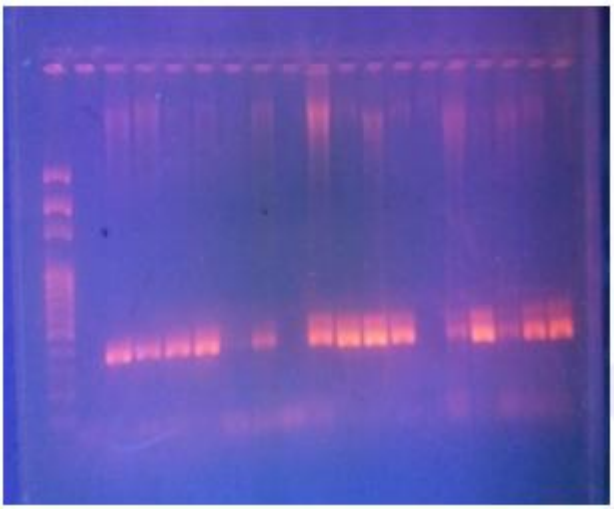

Fig. 1. nifH gene Amplification

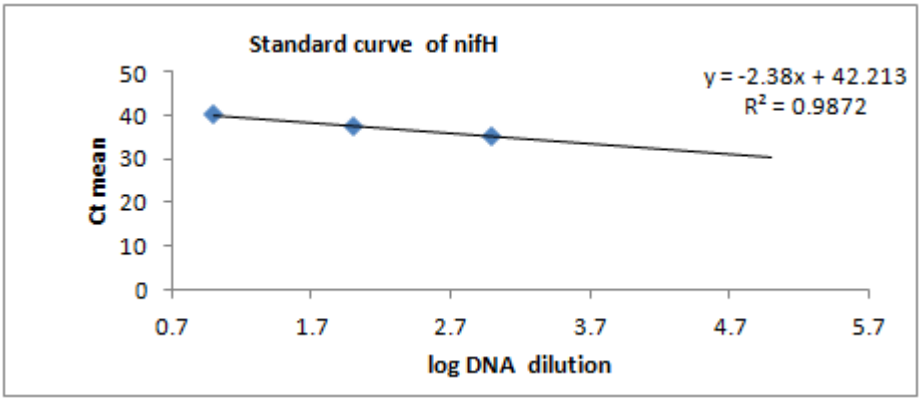

Fig. 2. Standard curve of NifH gene

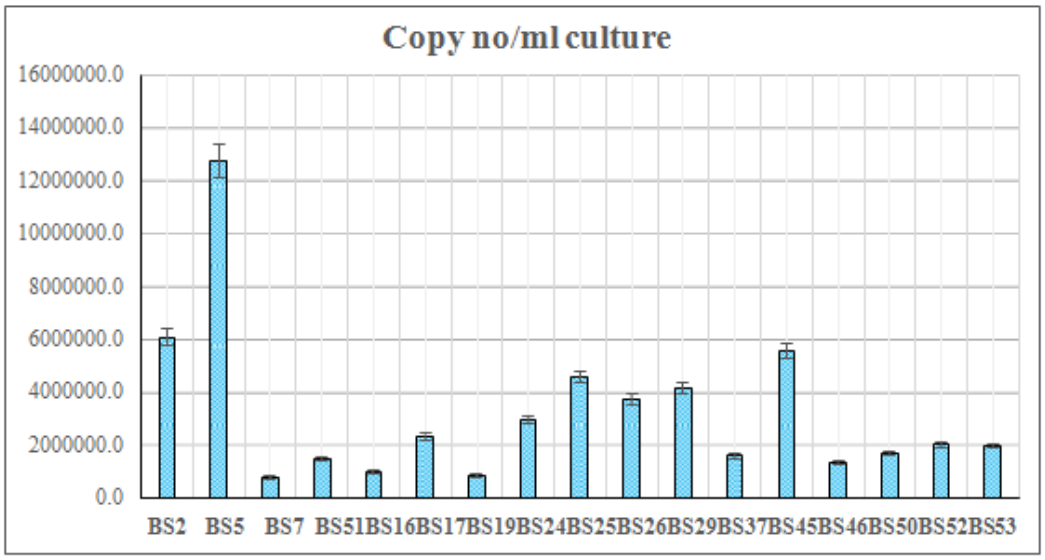

Fig. 3. NifH gene copy no/ml culture

other investigators [32]. From the foregoing results, it is suggested that PGPR are able to induce the production of IAA, solubilization of phosphorus, production of siderophore, resistance to pathogens and it could be concluded that those bacterial isolates that exhibit more than one PGPR characteristics *in-vitro* condition can be termed as polyvalent PGPR traits.

Molecular Identification of Potential Bacterial Isolate:

The molecular identification of the potential nitrogen fixing bacteria BS2 JGS, BS 5 JGS, BS25 BHU, BS29 ANG and BS45 GNJ were done by 16s r-DNA sequencing (Eurofins Pvt. Ltd). Consensus sequence of 16S r-DNA was generated from forward and reverse sequence data using aligner software. The consensus sequence was analyzed with BLAST search tool of NCBI Gene bank for the identification of the bacterial isolates. The homologous 16S r-DNA gene sequences of the selected strains were obtained from the microbial nucleotide databases through NCBI facility. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program. The Phylogenetic tree was constructed using MEGA 6.06 software and the results thus revealed that the bacteria BS2 JGS, BS25 BHU, BS29 ANG and BS45 GNJ belongs to genus *Klebsiella* and *Enterobacter* of the family Enterobacteriaceae and BS5 JGS belongs to genus *Rhizobium* of the family Rhizobiaceae. The 16S r-DNA genome sequence of all the for strain were submitted to NCBI Gene bank. As the isolates BS2 JGS, BS25 BHU, BS29 ANG and BS45 GNJ were not found to be *Rhizobium*, only BS5 JGS was selected for the further study. The 16S rRNA genome sequence of the *Rhizobium* sp. was submitted to GenBank and assigned with an accession number OK602 696.

Suppression of Fungal Phytopathogens (*in-vitro*) by Potent *Rhizobium* Isolate:

The isolate *Rhizobium* sp. BS 5 JGS was found to inhibit fungal phytopathogens *Fusarium oxysporum* ITCC 4998 and *Rhizoctonia solani* ITCC 2060, wherein 55.5% inhibition was observed in *Fusarium oxysporum* ITCC 4998 and 75.3% inhibition was observed in *Rhizoctonia solani* ITCC 2060.

Determination of Organic Acid Produced by the *Rhizobium* sp. using HPLC:

Estimation of organic acid production by the *Rhizobium sp*. BS5 JGS was done by using HPLC. On the basis of the set standard of organic acids, it was estimated that, the bacterial isolate was showing highest production of adipic acid, followed by tartaric acid, malonic acid, citric acid, ascorbic acid, phosphoric acid, oxalic acid and malic acid.

Behera et al. [33] reported similar findings while working on Phosphate solubilization and acid phosphatase activity of Serratia sp. isolated from mangrove soil of Mahanadi river delta, Odisha, India. During phosphate solubilization, various organic acids, such as malic acid (237 mg/l), lactic acid (599.5 mg/l) and acetic acid (5.0 mg/l) were detected in the broth culture through HPLC analysis.

Table 2. Plant growth promoting traits of the potent isolates

Test	Isolate No.				
	BS2 JGS	BS5 JGS	BS25 BHU	BS29 ANG	BS45 GNJ
Siderephore production	+	+	-	+	-
HCN production	-	-	-	-	-
NH3 production	+	+	+	+	+
IAA production	+	+	+	+	+
N2 fixation	+	+	+	+	+
Phosphate solubilization	+	+	-	+	-
Zinc solubilization	+	+	-	-	+
Potassium solubilization	-	+	-	+	-

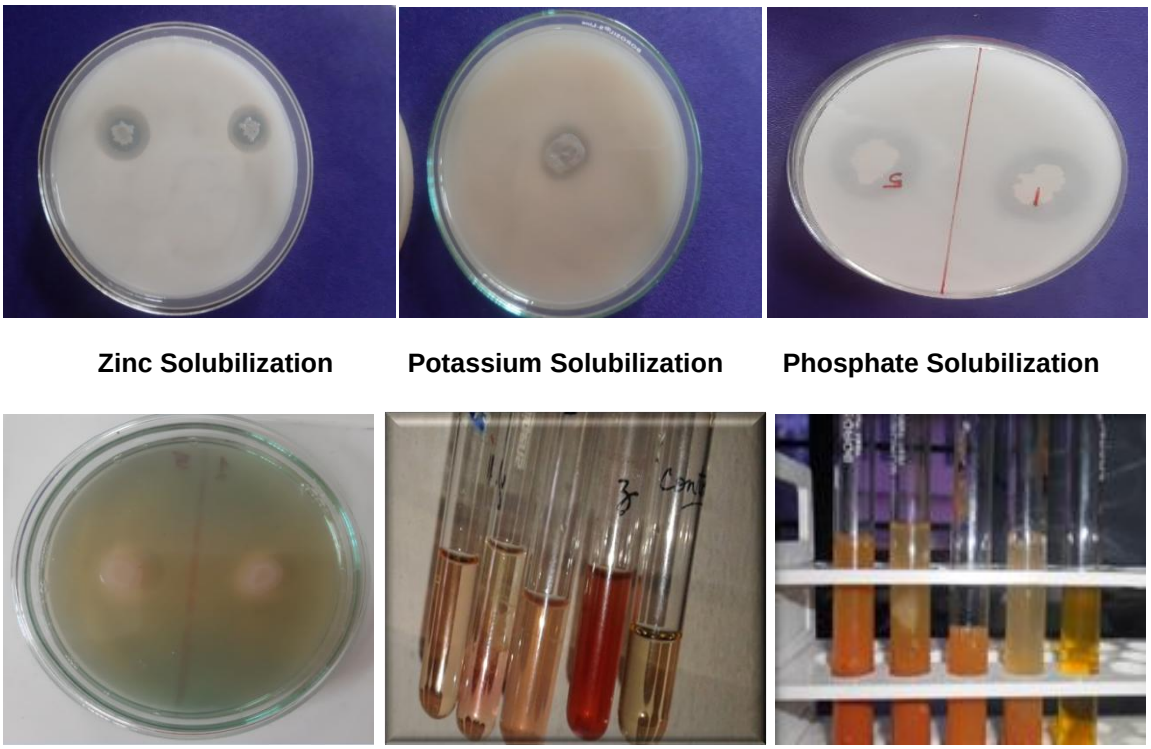

Fig. 4. Plant Growth promoting traits of the potential isolates

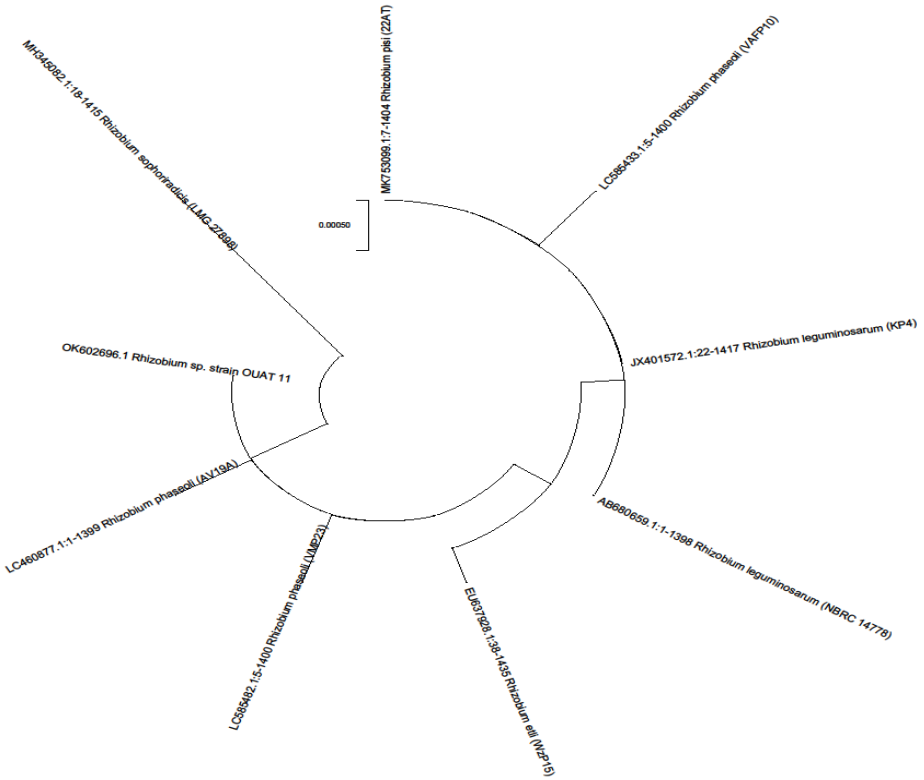

Fig. 5. Evolutionary analysis using 16s r-DNA sequences

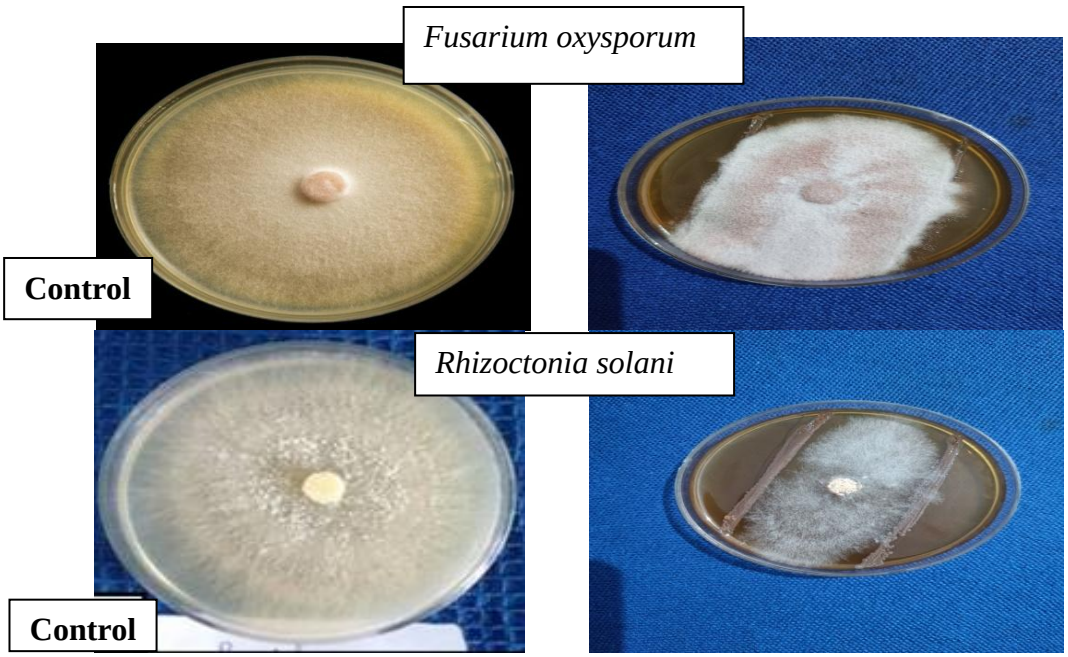

Fig. 6. Suppression of fungal phytopathogens by the potent *Rhizobium* sp

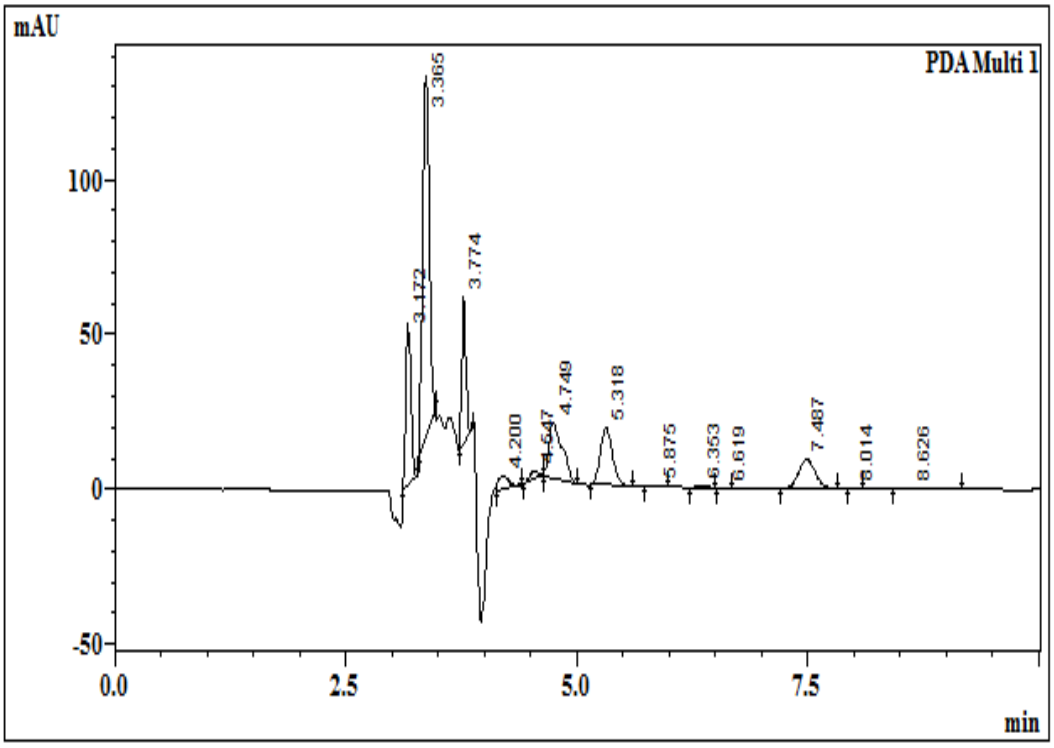

Fig. 7. HPLC Chromatograph image for Organic Acid estimation

Table 3. Types of organic acids detected through HPLC

Sample	Wavelength	Retention time	Area	Height	Area %	Organic acid	Concentration (in ppm)
Sample 1	218nm	3.172	201680.7	51763	13.7329	Tartaric acid	207.5713
Sample 1	218nm	3.365	584992.1	117977.7	39.8334	Ascorbic acid	39.91793
Sample 1	218nm	3.774	144267.5	47224.5	9.8235	Malonic acid	183.1734
Sample 1	218nm	4.2	31903.4	4278.3	2.1724	Phosphoric acid	34.5434
Sample 1	218nm	4.547	17050.8	2306.8	1.161	unidentified	-
Sample 1	218nm	4.749	180089.6	17842.9	12.2627	Citric acid	136.5306
Sample 1	218nm	5.318	167948.7	18562.9	11.436	Adipic acid	302.2673
Sample 1	218nm	5.875	812.4	117.6	0.0553	Oxalic acid	-
Sample 1	218nm	6.353	3081.4	376	0.2098	unidentified	-
Sample 1	218nm	6.619	539.3	111	0.0367	unidentified	-
Sample 1	218nm	7.487	124233.3	9526.9	8.4593	Malic acid	-
Sample 1	218nm	8.014	918.5	134.6	0.0625	unidentified	-
Sample 1	218nm	8.626	11078.2	557.7	0.7543	unidentified	-

Plate assay to estimate the solubility index by the *Rhizobium* isolate for Phosphorus, Zinc and Potassium:

Solubility index = [halozone diameter (mm) – colony diameter (mm)] / colony diameter (mm)

Quantification of Phosphate, Zinc and Potassium solubilisation by the bacterial isolate using ICP-OES: Quantification of Phosphorus, Zinc and Potassium was performed using ICP-OES where it was ascertained that the *Rhizobium* isolate was solubilizing Zinc much efficiently followed by Phosphorus and Potassium.

Evaluation of effective PGPR Rhizobium isolate on seed germination and growth of pulse plants by using Roll towel method using green gram seeds: On trial with green gram, the bacterial isolate with 100 seeds, in the germination paper, Percent germination with *Rhizobium* sp. BS5 JGS was even more than that of control. The percent of germination in control was 89% whereas it was 97.6% in *Rhizobium* sp. Root length and shoot length was also increased when seeds were pre treated with *Rhizobium* sp. BS5 JGS. In control, 10.53cm root length was recorded where as the length of root was 15.3cm in *Rhizobium* sp. BS5 JGS. Similarly, 17.9cm of shoot length was recorded in case of *Rhizobium* sp. BS5 JGS 12 as compared to 14.0cm in control. The *Rhizobium* sp. BS5 JGS isolate significantly increased the germination of seeds, root & shoot length of green gram seeds.

Table 4. Solubilization index by the *Rhizobium* isolate for Phosphorus, Zinc and Potassium

	Phosphorus	Zinc	Potassium
Solubility index	1.352	2.454	1.75

Table 5. Quantification of K, P and Zn through ICP-OES

Days of Incubation	mg/L (ppm)
0day K	0.992
2day K	1.522
4day K	1.786
6day K	1.838
0day Zn	0.060
1day Zn	1.182
2day Zn	2.747
3day Zn	5.743
4day Zn	5.964
0day P	0.146
1day P	0.170
2day P	0.622
3day P	1.640
4day P	2.883

**K= Potassium, Zn= Zinc, P= Phosphorus*

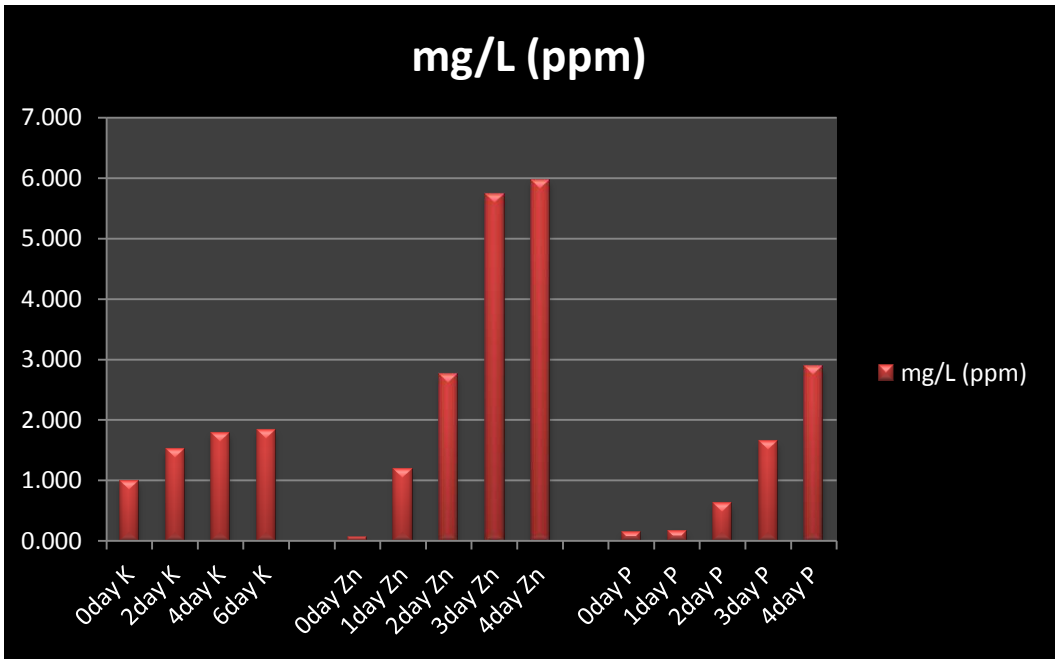

Fig. 8. Quantification of K, P and Zn through ICP-OES

Table 6. Effect of the potent bacterial isolate on seed germination of green gram using roll towel method

Isolate code	Germination%	Root length(cm)	Shoot length(cm)
Control	89 ±2.08	10.53±0.6	14.0±0.9
Rhizobium sp. BS5 JGS	97.6±1.45	15.3 ± 0.8	17.9±0.2

(Values are mean of three samples & ± represent SEM. Values are highly significant at p <0.05 by one-way ANOVA analysis)

Fig. 9. Effect of the potent bacterial isolate on seed germination of green gram using roll towel method

4. CONCLUSION

Fertilizers are essential components of modern agriculture because they provide essential plant nutrients. However, the overuse of fertilizers can cause unanticipated environmental impacts. In a view of the focus on organic farming, microbial diversity and soil health have gained considerable attention in recent years. Applications of organic manures directly and/or indirectly increased soil microbiome that plays a pivotal role in maintaining soil fertility and increasing productivity. From the present study it was concluded that *Rhizobium* sp. BS5 JGS exhibited plant growth-promoting traits like Phosphate solubilization, Siderophore production, IAA production, Zinc and Potassium solubilization and nitrogen fixation. The isolate exhibited an efficient nitrogenase activity by having maximum copy numbers of nifH gene. It was found to significantly increase the germination percentage and growth parameters in seed germination studies.

Moreover, the *Rhizobium* strain exhibited antagonistic suppression of two most common fungal phytopathogens *Fusarium oxysporum* and *Rhizoctonia solani.*

Furthermore, application of formulation of this potent *Rhizobium* strain can provide plant growth as well as effective, economical and practical way of plant protection through suppression of pathogens. So, the use of this *Rhizobium* isolate as biofertilizers is a novel approach to replenish and replace the chemical fertilizers and pesticides for sustainable agriculture in India and PGPR technology should be utilized along with appropriate levels of fertilization to achieve maximum benefits in terms of fertilizer savings and better growth.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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