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Isolation and initial characterization of diazotrophic plant growth promoting rhizobacteria (PGPR) from rice rhizosphere of Parsa and Bara district of Nepal

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Abstract

Diazotrophic bacteria were isolated from the rhizosphere of rice plants in two districts (Parsa and Bara) of Nepal. The rate of nitrogenase enzyme activity based on acetylene reduction assay showed remarkable variation in these isolates which ranged from 0.69 to 1.63 µmol C_2H_4 formed/mg protein/h. Their plant growth promoting characters were also analysed. It was observed that 64.3% of them showed IAA production, 32.1% phosphate solubilization, 53.6% siderophore production whereas 10.7% isolates showed all the plant growth promoting characters. Based on biochemical characterization and carbon source utilization they were clustered in 6 clusters. It is hereby reported that species of *Pseudomonas, Klebsiella, Azotobacter* and *Agrobacterium* were predominantly present in this region especially in the rhizosphere of rice plants.

Key-Words: Diazotrophic bacteria, Plant growth promoting rhizobacteria (PGPR), Nitrogen fixation, IAA production, Phosphate solubilisation, Siderophore production

Introduction

Plant-associated bacteria that are able to colonize roots are called rhizobacteria and can be classified into beneficial, deleterious, and neutral groups on the basis of their effects on plant growth. Beneficial rhizobacteria that stimulate plant growth are usually referred to as plant growth promoting rhizobacteria or PGPR [1]. PGPR are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots. They can improve the extent or quality of plant growth by direct and/or indirect methods. In last few decades, a large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligens, Arthobacter, Burkholderia, Bacillus* and *Serratia* have been isolated and reported to enhance plant growth [1, 2]. The direct plant growth promotion by PGPR entails either providing the plant with a plant growth promoting substances that are synthesized by the bacterium or facilitate the uptake of certain plant nutrients from the environment. The indirect promotion of plant growth occurs when PGPR minimize or prevent the deleterious effect of one or more phytopathogenic micro-organisms and help plants to fight against the environmental stresses.

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Several PGPR have been found successful in growth promotion in certain crops such as canola, soybean, lentil, pea, wheat and radish [3, 4]. The enhancement of plant growth by PGPR indicates their potential as bio-fertilizers in the field of agriculture. Though the importance of PGPR is well understood, but efficient PGPR are lacking. Parsa and Bara districts are better rice producing district in Nepal but, screening of rhizobacteria for the search of efficient PGPR have not been done. Keeping this objective in mind the present investigation was performed and their plant growth promoting abilities were evaluated after screening and isolation of diazotrophic plant growth promoting rhizobacteria (PGPR) from the rhizosphere of rice plants of Parsa and Bara districts of Nepal.

Material and Methods

Soil Sample Collection and Isolation of Bacteria

Rice (*Oryza sativa L.*) plants of different fields of Parsa and Bara districts of Nepal were selected for the study. The locations of the fields showing latitude and longitude of the collection sites are shown in Table 1. Soil samples from rhizosphere of rice plants were collected carefully by uprooting the root system and placed in a sterile polythene bag for transport and stored at 4°C. 1.0 gram of rhizospheric soil was suspended in 1.0 mL of sterile DDW in sterile test

tube and was mixed properly. After 1 h of sedimentation, 1.0 mL of water was taken from the tube and it was mixed in another 9.0 mL of sterile DDW for dilution. 1.0 mL of bacterial suspension was further diluted in another sterile test tube containing 9.0 mL DDW. In this way it was diluted 10^{-7} dilution. 100 µL of suspension was kept in solid JNFb$^-$ agar plate and spreading was done. It was incubated for 3 days at 30^{o}C in BOD incubator and morphologically different colonies appeared on the plates were isolated, sub cultured and enriched in JNFb$^-$ medium [5] devoid of combined nitrogen. The JNFb$^-$ medium comprises 0.5% (w/v) of Malic acid, 0.06% (w/v) of K_2HPO_4, 0.18% (w/v) of KH_2PO_4, 0.02% (w/v) of $MgSO_4.7H_2O$, 0.01% (w/v) of NaCl, 0.02% (w/v) of $CaCl_2.2H_2O$, 0.0002% (w/v) of $Na_2MO_4.2H_2O$, 0.45% of KOH, 5 mL/L of bromothymol blue (0.5% in 0.2 N KOH) and 4 mL/L of Fe-EDTA (1.4%).

Acetylene reduction assay

Nitrogenase activity was estimated by acetylene reduction assay [6]. Overnight LB medium grown cultures (1.5 mL) was spun and washed carefully with sterilized DDW to remove sources of combined nitrogen, if any, and then suspended in 100 µL of PBS (phosphate-buffered saline). 10 µL from this inoculum was added in 3 mL semi-solid (0.15 % agar w/v) JNFb$^-$ medium in a 7 mL vacutainer tube (Becton-Dickinson, Rutherford, NJ, USA) and grown for 3 days. Thereafter pure acetylene gas was injected in each tube by a hypodermic syringe to attain 10% final concentration. All the assays were performed at 30^{o}C without shaking. The ethylene formed was analyzed in a 5700 Nucon Gas Chromatograph (Nucon Engineers Ltd., New Delhi) fitted with Porapak R column and flame ionization detector. N_2 was used as the carrier gas. Nitrogenase activity (acetylene reduction) was expressed in terms of µ moles C_2H_4 /mg protein /h.

Estimation of IAA by colorimetric method

The production of indole-3-acetic acid (IAA) was tested by colorimetric method of Gordon and Weber, 1951 [7]. Cultures were grown in JNFb$^-$ liquid medium with or without tryptophan (100 µg/mL) at 30^{o}C with shaking at 80 rpm for three days. 1.5 mL culture was centrifuged at 8000 rpm for 5 min at desired time interval and pellet was discarded and supernatant retained. To 1 mL supernatant, 2 mL IAA reagent (1 mL of 0.5 M $FeCl_3$ was mixed in 50 mL of 35% $HClO_4$) was added. The sample was incubated at room temperature for 25 min. The optical density of the samples was recorded at 530 nm (blank from respective medium was used). The amount of IAA was quantified with standard of pure IAA prepared separately.

Test of Phosphate Solubilization

All isolates were first screened on Goldstein agar plates for phosphate solubilization as described by Goldstein, 1987 [8]. Quantitative analysis of phosphate solubilization was performed as per the method of Mehta and Nautiyal, 2001 [9]. The test isolates were inoculated in Nautiyal phosphate solubilization broth. The Nautiyal medium comprises 1.0% (w/v) glucose, 0.5% (w/v) $Ca_3(PO_4)_2$, 0.5% (w/v) $MgCl_2$-$6H_2O$, 0.025% (w/v) $MgSO_4$ -$7H_2O$, 0.02% (w/v) KCl and 0.01% (w/v) $(NH_4)_2SO_4$. The pH was adjusted 7.0. The culture was incubated for 3 days at 30 ± 2^{o}C. The cultures were harvested by centrifugation at 10,000 rpm for 10 min. The culture supernatant thus obtained was used for quantitative assays. The optical density was measured at 600 nm with spectrophotometer.

Test of Siderophore Production

The CAS (Chrome Azurol S) assay [10] is the universal chemical assay for the detection of siderophore and is based on a siderophore's high affinity for ferric iron. CAS plates are blue in colour because chrome azurol S dye complex is formed with ferric iron. When siderophore is present, the following reaction occurs, which releases the free dye, which is orange in colour.

Fe^{3+} - dye (blue) + siderophore ⟶ Fe^{3+} - siderophore + dye (orange)

Accordingly, CAS agar plates were prepared containing: 1 mM Chromeazurol-S, 10 mL $FeCl_3.6H_2O$ (1 mM) made in 10 mM HCl, and N, N-cetyl trimethyl ammonium bromide (2 mM) (CTAB). This was autoclaved separately and added to 300 mL of JNFb$^-$ medium containing 2 µM $FeCl_3$ replacing the Fe-EDTA which is the usual ingredient of the medium. 10 mL of iron starved (JNFb$^-$ medium grown) culture was inoculated as spot inoculation on the CAS agar plate and incubated for 72 h at 30^{o}C. Yellow to orange halo zone appearing around the colonies was recorded as positive test for siderophore production.

Estimation of siderophore concentration

The isolates were grown in JNFb$^-$ liquid medium without any iron source to create iron starvation. Iron starved isolates were inoculated in JNFb$^-$ liquid medium containing 2µM $FeCl_3$ in place of Fe-EDTA. Estimation of siderophore was made after 3 days of incubation at 30^{o}C. For this culture supernatant was recovered by centrifugation at 8000 rpm for 5 min. 0.5 mL supernatant containing less than 7.5 nmol of iron chelator was mixed with 0.5 mL of CAS assay solution. 2,3-dihydroxybenzoic acid (DHBA) or

catechol was used as positive control and un-inoculated medium as negative control. Absorbance was measured at 630 nm.

Biochemical Characterization

The Gram reaction was performed as per standard protocol. Physiological and biochemical characterizations of the bacterial isolates such as catalase, urease, nitrate reduction, IMViC and carbon source utilization were examined according to the standard methods [11]. The isolates were identified accordance with the Bergey's manual of determinative bacteriology [12].

Results and Discussion

Isolation of Diazotrophic Rhizospheric Bacteria

Altogether 28 bacterial isolates were isolated from rhizospheric soil sample of different locations of 14 rice fields of different agro-climatic conditions and cultivation pattern of two districts of Nepal employing standard enrichment techniques using $JNFb^-$ solid agar agar medium. All these isolates were routinely sub-cultured on solid $JNFb^-$ agar-agar plates so as to ensure their diazotrophic character. When inoculum of dilutions was spread on to solid agar-agar $JNFb^-$ medium, a number of discrete colonies of bacteria appeared after 3-4 days of incubation. Maximum number (3 morphotypes) of morphologically distinct colonies was noted in the sample of Sirisiya, Birganj of Parsa district (PN-4) and the minimum (1) in the samples of Khalwa tola, Birganj of Parsa district (PN-1) and other all samples showed 2 morphotypes. Selection of various morphotypes was performed on the basis of size, shape, colour and elevation of colonies. Table 1 shows that out of 14 soil samples collected from different rice fields, the highest number of bacterial population (36.9×10^7) was observed in the sample of Parwanipur agriculture farm (BN-7) and the lowest (14×10^4) in sample of Pipara V.D.C. namely BN-9. Table 2 shows acetylene reduction assay (nitrogenase activity) of all the isolates. It is evident from the results that the rate of nitrogenase activity differs significantly among all the isolates. Among all the isolates BN-4A showed highest nitrogenase activity (1.63 µmol C_2H_4 formed/mg protein/h) and the lowest activity was detected in BN-1B and BN-5A (0.69 µmol C_2H_4 formed/mg protein/h). It is interesting to note that the rate of nitrogenase activity varied markedly even in isolates obtained from the same locations. However results clearly show that all these isolates can fix N_2 under reduced O_2 tension though they are aerobic N_2 fixers (Table 2).

In the present study, we have made an attempt to isolate diazotrophic bacteria from the rhizosphere of rice plants of different fields located Bara and Parsa of Nepal. It is evident from our results that there is presence of diverse types of diazotrophic bacteria in almost all the rice fields. The isolation and enrichment of diazotrophic bacteria were based on repeated growth in the medium devoid of any combined nitrogen sources. The bacterial colonies appearing on plates were differentiated and selected on the basis of morphological characters and it was observed that in general each soil sample contains 1 to 3 morphotypes of colonies. Presence of rhizobacteria with N_2 fixation ability in the rhizosphere of various plants has been reported from various crop plants. A number of rhizospheric diazotrophs were isolated in the 1960s to 1970s, but their contribution in the nitrogen nutrition is still under debate [13]. The specificity of the plant-bacteria interaction depends upon soil conditions, which can alter contaminant bioavailability, root exudates composition, and nutrient levels of the rhizosphere. Our study shows that the population size of bacteria of different rice fields differ which might be due to differences in genotypes of plants and partly due to soil types.

Test of Plant Growth Promoting Activities

IAA production, phosphate solubilization and siderophore production are considered to be the major plant growth promoting features of any bacteria. Accordingly tests for these characters were made in all the isolates. It is evident from the data of Table 3 that out of 28 isolates, 18 (64.3%) showed positive test for IAA production. IAA production occurred solely in liquid $JNFb^-$ medium supplemented with tryptophan (100 µg/mL), there was no production in medium lacking tryptophan. With a view to optimize the concentration of tryptophan for IAA production, varying concentrations (ranging from 50 to 500 µg/mL) were used in the medium and it was observed that optimum IAA production was achieved at 100 µg/mL. Among all the isolates BN-2A showed highest IAA production (16.36 ± 0.3 µg/mg dry weight) and PN-5A showed the lowest level (0.73 ± 0.4 µg/mg dry weight) (Table 3). Out of 28 isolates, 9 (32.1%) produced halo-zones around the growing colony on Goldstein solid medium, whereas other 19 (67.9%) either showed growth without any halo-zone formation or failed to grow.

Efficiency of phosphate solubilization was further confirmed by measuring the level of solubilized P in the liquid Nautiyal medium (Table 3). It is evident that BN-4A, an isolate from Bara district of Nepal showed highest P solubilization (33.17 ± 5.6 µg/mg dry weight) and BN-9C, an isolate from Bara district

also showed lowest P solubilization (11.52 ± 4.5µg/mg dry weight).

Siderophore production was detected by means of CAS agar plate assay where blue colour of medium changed to yellow/orange around the growing colonies. On the basis of yellow/orange halo zone formation, 13 isolates (28.2%) showed positive test for siderophore production. Quantitative estimation showed that the highest siderophore (22.22 ± 3.45 µg/ mg dry weight) production occurs in the isolate BN-2B whereas the lowest (8.5 ± 1.65 µg/ mg dry weight) level was in PN-4D (Table 3).

Plant growth promoting tests of all these putative diazotrophic isolates revealed that 21 isolates showed at least one character i.e., IAA production, phosphate solubilization and siderophore production but 7 isolates failed to show any one of these three characters. Accordingly, 21 isolates showing plant growth promoting characteristics were selected for further study (Table 4). Bacterial isolates that produce relatively higher amount of IAA may affect root growth adversely; many plant pathogenic bacteria, viz., *Pseudomonas savastonoi* [14], *Agrobacterium tumefaciens* and *A rhizogenes* [15], also produce phytohormones leading to pathogenesis. The bacteria reported in this study produced moderate range of IAA (1.75 to 16.35 µg/mg dry weight) and thus may be treated as potential PGPR. Plant growth promotion observed with bacteria that produce moderate levels of IAA includes *Azospirillum* sp., *Alcaligenes faecalis*, *Klebsiella* sp., *Enterobacter cloacae*, *Acetobacter diazotrophicus*, *Rhizobium* [14], *Klebsiella oxytoca* strain GR-3 [16] and *Pseudomonas putida* [17]. Our results are in agreement with the above reports both in terms of level of IAA production and bacterial isolates tentatively identified. Most of the agricultural soils in various parts of India contain 50 kg/ha of phosphorous, primarily in the unavailable form of precipitated tricalcium phosphate ($Ca_3(PO4)_2$) in which only 2-3 ppm phosphorous is available to rice [18], whereas the exact proportion of tricalcium phosphate in the soil of Nepal is not available in the literature. It is expected that the level of available P may be more or less similar to those present in soil of India. Many bacteria are well known to dissolve bound phosphates such as calcium triphosphate, hydroxyapatite and rock phosphate, and enhance the availability of phosphorous for microbial and/or plant growth [19]. The highest P-solubilizing isolate BN-4A (isolated from Bara district) shows 2-3 times more P-solubilizing activity than other isolates and posses all the plant growth potentials tested in this investigation seems to be a good isolate for use as biofertilizer. P solubilization by rhizospheric bacteria is useful character since they can partly meet P demand of the plants. Several workers have reported that seed or soil inoculation with phosphate-solubilizing bacteria improves solubilization of fixed soil phosphorous and/or applied phosphates, resulting in higher crop yields [18].

Majority of the soil contains enough amount of iron (1-6%) but most of them are in ferric form, which is insoluble and thus not accessible to the plants and microorganisms. Many microorganisms including bacteria and fungi have developed system for the synthesis of low molecular weight organic compounds, siderophore, which efficiently solubilize and transport ferric iron [20]. We have analyzed all the isolates for their iron chelating properties and observed that out of 28 isolates, 15 isolates produced siderophore in iron limiting condition. It has been demonstrated that synthesis of siderophore is induced when iron is depleted from the culture medium [21]. Our finding is in agreement with earlier report since we also observed induction under iron depleted condition. A number of diazotrophic bacteria and PGPR such as species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Klebsiella*, *Enterobacter*, *Xanthomonas* and *Serratia* were isolated by different workers from different agro-ecosystems. All the above isolates promoted plant growth [22]. Similar to above report we also found species of *Agrobacterium, Klebsiella, Microbacterium, Pseudomonas, Azotobacter* and *Serratia*. All the isolates were capable to fix N_2, 64.3% was IAA-producing, 32.1% phosphate solubilizing and 28.2% siderophore producing. A number of isolates showed more than one plant growth promoting characters. Three isolates (*Pseudomonas* sp. strain PN-4D, *Agrobacterium* sp. strain BN-2A and *Klebsiella* sp. strain BN-4A) which are 10.7% of total population showed all the PGP characters and accordingly categorized as efficient PGPR. Presence of the above characters may be responsible for plant growth enhancement.

Biochemical Tests

It is evident with the table 4 that all isolates showed gram negative test. Motility test showed that 19 (90.5%) isolates were motile and 2 (9.5%) were non-motile. The biochemical test revealed that all the isolates were positive for catalase and nitrate reductase, however 19 (90.5%) showed positive test for urease and motility. Methyl red test revealed that out of 21 isolates, 10 (47.6%) was methyl red positive and 11 (52.4%) was negative. Simmon's citrate test showed that 18(85.7%) isolates were positive and 3 (14.3%) was negative (Table 4). For

lactose fermentation test all the isolates were grown on Mac Conkey's agar plate. Results showed that out of 21 isolates, 8 (38.1%) was found positive for lactose fermentation and other 13 (61.9%) was negative (Table 4).

Based on carbon source utilization and biochemical characterization tentative grouping of all the isolates could be made in six clusters and accordingly generic assignment was proposed. The cluster I contains 3 (14.3 %) isolates comprising BN-1A, BN-1C and BN-2A were rod shaped, gram's negative, motile, circular colonies, smooth, non-pigmented, catalase and urease positive and certain isolates failed to grow with glucose. They showed citrate utilization and salt tolerance up to 5.0 % (w/v) and were methyl red negative, nitrate reductase positive, IAA producing, P-solubilizing, N_2-fixing and siderophore producing. All these features resemble to the genus *Agrobacterium*. Cluster II contains 6 isolates namely, PN-4D, BN-2B, BN-2C, BN-2D, BN-7C and BN-9C were straight or slightly curve rods, gram's negative, motile, some species were non-motile, aerobic and catalase positive. These features resemble to those of the genus *Pseudomonas*. Morphological features and biochemical tests of 6 isolates namely, BN-3C, BN-3D, BN-4A, BN-5A, BN-5B and BN-5C showed close similarity with *Klebsiella* sp. and they are kept in cluster III. These isolates were rod shaped gram-negative, non-motile and facultative aerobes. Optimum growth occurred at 37^{o}C, glucose and other carbohydrates were catabolized with the production of acid and gas. They were catalase and urease positive. Indole, methyl red, Voges-Proskaur and Simmon's citrate reactions varied among different isolates. 2 isolates such as PN-5A and PN-7D were straight rods, gram's -negative, usually motile, facultative anaerobes having both respiratory and fermentative types of metabolism. They grew well at 30-37^{o}C. Glucose and other carbohydrates were catabolized with production of acids and often gas. Methyle red test varied, Simmon's citrate test was positive, Voges-Proskaur test was usually positive and urea was not hydrolyzed. Based on above features they are kept in cluster VI and it appears that these isolates may belong to the genus *Serratia*. One isolate namely BN-6B showed similarity with the genus *Azotobacter* and that was grouped in separate cluster V. This isolate was large rods or ovoid cells, occurred singly or in pairs or clumps and sometimes in chains of varying lengths. They were gram's-negative, motile or non-motile, aerobic, but could grow under low oxygen tension, catalase positive, grew on broad pH range (4.8- 8.5) and showed salt tolerance up to 5% (w/v). Biochemical tests were not enough to assign generic status to 3 isolates (PN-3D, PN-4B and PN-7C). Molecular characterization is needed to identity of all these isolates. Since these isolates were not efficient PGPR, we did not focus our attention on these isolates and kept as unidentified isolates.The bacterial diversity of rice rhizosphere of Parsa and Bara districts was analyzed at the first time that showed that *Pseudomonas*, *Klebsiella*, *Serrattia*, *Azotobacter*, *Agrobacterium* are mainly dominant bacterial population in the rice rhizosphere of these regions.

Conclusion

In conclusion, we hereby report that species of *Pseudomonas, Klebsiella, Azotobacter* and *Agrobacterium* were predominantly present in the rhizosphere of rice plants of both districts of Nepal. Based on their potentials for nitrogen fixation, IAA production, P- solubilization, siderophore production, their efficiency as bio-fertilizer have been checked on pot/field level trial which has been communicated for publication elsewhere.

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Table 1: Details of sample collection sites showing their coordinate ((Latitude & Longitude) and CFU/g of soil sample

District	Sample ID	Locality of collection	(Latitude & Longitude ± 1.0')	Colony forming units/g soil	No. of Isolates
Parsa	PN-1	Khalwa Tola, Birganj	27°5′57″N, 84°45′0″E	20 X 10^5	1
	PN-3	Khalwa Tola, Birganj	27°5′57″N, 84°45′0″E	4.8 X 10^5	2
	PN-4	Sirisiya, Birganj	27°0′0″N, 84°48′50″E	80 X 10^5	3
	PN-5	Sirisiya, Birganj	27°0′0″N, 84°48′50″E	24 X 10^4	2
	PN-6	Sirisiya, Birganj	27°0′0″N, 84°48′50″E	39 X 10^4	2
	PN-7	Chandal Chawk	27°0′44″N, 84°48′42″E	30.4 X 10^5	2
Bara	BN-1	Parwanipur Agriculture Farm	27°4'59"N, 84°52'59"E	20.8 X 10^7	2
	BN-2	Parwanipur Agriculture Farm	27°4'59"N, 84°52'59"E	18.9 X 10^7	2
	BN-3	Parwanipur Agriculture Farm	27°4'59"N, 84°52'59"E	8.7 X 10^5	2

	BN-4	Parwanipur Agriculture Farm	27°4'59"N, 84°52'59"E	28.6 X 10^7	2
	BN-5	Parwanipur Agriculture Farm	27°4'59"N, 84°52'59"E	26.1 X 10^7	2
	BN-6	Pipara V.D.C.	27°3′10″N, 84°52′28″E	10.7 X 10^5	2
	BN-7	Parwanipur Agriculture Farm	27°4'59"N, 84°52'59"E	36.9 X 10^7	2
	BN-9	Pipara V.D.C.	27°3′10″N, 84°52′28″E	14 X 10^4	2

Table 2: Nitrogenase activity of various isolates

Isolates	Nitrogenase activity (μmol C_2H_4/mg protein/h)
PN-1A	0.78 ± 0.09
PN-3B	0.73 ± 0.12
PN-3C	0.76 ± 0.08
PN-3D	0.88 ± 0.07
PN-4B	0.84 ± 0.08
PN-4D	1.33 ± 0.5
PN-5A	0.93 ± 0.15
PN-6D	0.82 ± 0.13
PN-7C	0.73 ± 0.08
PN-7D	0.94 ± 0.12
BN-1A	0.97 ± 0.07
BN-1B	0.69 ± 0.09
BN-1C	0.86 ± 0.16
BN-2A	1.42 ± 0.13
BN-2B	1.16 ± 0.14
BN-2C	0.98 ± 0.13
BN-2D	0.95 ± 0.14
BN-3C	0.86 ± 0.08
BN-3D	0.79 ± 0.07
BN-4A	1.63 ± 0.15
BN-5A	0.69 ± 0.09
BN-5B	0.79 ± 0.08
BN-5C	0.96 ± 0.06
BN-6B	0.87 ± 0.07
BN-7A	0.91 ± 0.08
BN-7C	0.94 ± 0.12
BN-9C	1.02 ± 0.13
BN-9D	0.84 ± 0.09

Nitrogenase activity was tested in $JNFb^-$ semi-solid (0.15%) medium. Cultures were preincubated for 6 h with C_2H_2 for estimation of C_2H_4 formation, # Results are based on average of three experiments conducted under identical condition

Table 3: **Rate of IAA production, P solubilization and siderophore production in various isolates**

Isolates	IAA production (μg/mg dry weight)	Phosphate solubilization (μg/mg dry weight)	Siderophore production (μg/mg dry weight)
PN-1A	-	-	-
PN-3B	-	-	-
PN-3C	-	-	-
PN-3D	3.17 ± 0.65	-	14.13 ± 2.15
PN-4B	5.72 ± 0.75	-	10.84 ± 2.68
PN-4D	1.92 ± 0.25	17.47 ± 4.2	8.5 ± 1.65

PN-5A	0.73 ± 0.4	-	8.76 ± 1.38
PN-6D	-	-	-
PN-7C	-	12.47 ± 2.5	8.92 ± 2.95
PN-7D	1.39 ± 0.2	-	9.73 ± 2.36
BN-1A	1.62 ± 0.2	15.66 ± 2.35	-
BN-1B	-	-	-
BN-1C	1.75 ± 0.25	14.27 ± 3.5	-
BN-2A	16.36 ± 0.3	20.78 ± 4.5	18.07 ± 2.89
BN-2B	3.99 ± 0.6	-	22.22 ± 3.45
BN-2C	5.11 ± 0.5	-	19.47 ± 2.36
BN-2D	2.65 ± 0.48	-	18.92 ± 2.55
BN-3C	2.69 ± 0.65	-	15.63 ± 2.67
BN-3D	6.26 ± 0.5	-	15.38 ± 3.92
BN-4A	4.67 ± 0.6	33.17 ± 5.6	17.78 ± 2.44
BN-5A	4.87 ± 0.75	-	18.77 ± 2.66
BN-5B	5.23 ± 0.85	-	19.61 ± 2.38
BN-5C	5.09 ± 0.65	12.23 ± 3.7	-
BN-6B	2.34 ± 0.55	-	-
BN-7A	-	-	-
BN-7C	-	12.17 ± 3.5	-
BN-9C	-	11.52 ± 4.5	-
BN-9D	-	-	-

For IAA production, cultures were grown in JNFb- liquid medium containing 100 µg/mL tryptophan for 3 days and thereafter IAA estimation was made, # P solubilization was tested after 3 days of growth in Nautiyal liquid medium, # Siderophore was estimated after 3 days growth of culture in JNFb- liquid medium containing 2µM of $FeCl_3$ in place of Fe-EDTA, # Results are based on average of three experiments conducted under identical conditions ± standard deviation

Table 4: Details of biochemical characterizations of various isolates

Parameters	Cluster-1	Cluster-2	Cluster-3	Cluster-4	Cluster-5	Cluster-6
Number of Bacteria	3	6	6	2	1	3
Gram reaction, Cell shape	- (3), rods	- (6), rods	- (6), rods	- (2), rods	- (1), rods	- (3), rods, cocci
Motility	+ (3)	+ (6)	+ (5) /- (1)	+ (2)	+ (1)	+ (2) /- (1)
Catalase	+ (3)	+ (6)	+ (6)	+ (2)	+ (1)	+ (3)
Urease	+ (3)	+ (6)	+ (6)	+ (2)	+ (1)	+ (1) /- (2)
Nitrate reductase	+ (3)	+ (6)	+ (6)	+ (2)	+ (1)	+ (3)
Nitrogen fixation	+ (3)	+ (6)	+ (6)	+ (2)	+ (1)	+ (3)
P-solubilization	+ (3)	+ (3) /- (3)	+ (3) /- (3)	- (2)	- (1)	-(3)
Siderophore production	+ (2) /- (1)	+ (3) /- (3)	+ (6)	+ (2)	- (1)	+ (2) /-(1)
IMViC						
Indole production	+ (3)	+ (6)	+ (4) /- (2)	+ (2)	+ (1)	+ (2 /- (1)
Methyle red	- (3)	+ (3)/- (3)	+ (6)	- (2)	+ (1)	- (3)
Voges-Proskaur	+ (3)	+ (3)/- (3)	- (6)	+ (2)	- (1)	+ (3)
Citrate(Simmons)	- (3)	+ (6)	+ (6)	+ (2)	+ (1)	+ (3)
Lactose fermentation	- (3)	- (6)	+ (6)	+ (2)	- (1)	- (3)
Growth on *Azotobacter* agar	+ (2) /- (1)	- (6)	+ (6)	- (2)	+ (1)	- (3)

Cluster 1 (*Agrobacterium* sp.): BN-1A, BN-1C and BN-2A, Cluster 2 (*Pseudomonas* sp.)**:** PN-4D, BN-2B, BN-2C, BN-2D, BN-7C and BN-9, Cluster 3 (*Klebsiella* sp.): BN-3C, BN-3D, BN-4A, BN-5A, BN-5B and BN-5C, Cluster 4 (*Serratia* sp.): PN-5A and PN-7D, Cluster 5 (*Azotobacter* sp.): BN-6B, Cluster 6 (Unidentified): PN-3D, PN-4B and PN-7C, - Negative; + Positive; *Figures in parenthesis indicate number of strain