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Isolation, characterisation and screening of *Rhizobium* from leguminous plant

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Abstract
Enrichment of nutrients in soil by nitrogen fixing bacteria particularly in legumes is an ancient fact know to everyone. *Rhizobium* sp. plays a key role in agriculture by fixing atmospheric nitrogen to the plants. The present study describes isolation; characterization and screening of *Rhizobium* sp. 83 *Rhizobium* sp. were isolated from root nodules of Pigeon pea plant supplemented with flyash. The isolates were characterized morphologically, biochemically, physiologically and further screened for PGPR activity by performing phosphate solubilization test, IAA test, ammonia production, HCN production, siderophore production and nitrate reduction test. *Rhizobium* isolated BUDR13 and BUDR27 were found to be the highly efficient strains.

Keywords: *Rhizobium*, Nitrogen fixation, flyash, phosphate solubilization, siderophore production

Introduction
Rhizobium Prokaryotic Organisms have the ability to reduce the atmosphere nitrogen fixation of elemental nitrogen in the atmosphere by the microorganisms through a reductive process into ammonia is called as biological nitrogen fixation (BNF). BNF account for about 78% of the total N_2 fixed in the biosphere. The ability to reduce atmosphere N_2 is restricted only to bacteria which are belonging to the diverse groups. *Rhizobium* are the first group of organisms realised for its potential of N_2 fixation. *Rhizobium* Sp. belongs to plant growth promoting bacteria (PGPR) group are the beneficial symbionts that enhance the plant growth. *Rhizobium* bacteria are known to participate in many biological activities such as biological control of plant pathogens nutrient cycling and seedling/plant growth (Wu *et al*, 2006) [65] (Dubey *et al*., 2001) [68] Fly ash is a coal combustion product which chiefly consist of components like SIO_2, Al_2o_3, Fe_2o_3 and occasionally cao (lime).

Materials and Methods
For the isolation of *Rhizobium* healthy root nodules of the pigeon pea plant were collected from the fields nearby Raichur thermal power station (RTPS). The roots were washed thoroughly with sterile distill water for 10 seconds and nodules which were obtained got surface sterilized in ethanol of 95% and again washed in sterile distilled water for few seconds for about 7 times and then nodule suspension was prepared using pestle and mortar the roots nodules were smashed to extract more milky white substances of bacteroids with the help of phosphate buffer solution. After the extraction of bacteroids solution from the roots nodules serial dilution plate count technique was followed. To get the growth of the *Rhizobium* bacteria about 2gms of serially diluted nodule and root rhizosphere soil amended with fly ash, collected from the dumped site of Raichur thermal power station were diluted in 10ml of water in a test tube which served as stock solution. Remaining nine test tubes were filled with 9ml of of sterilized distill water with the help of pipettes yielded 10^{-1} dilutions and the series continued upto 10^{-9} dilutions. Then the serial dilution 10^{-2} and 10^{-3} were choosen. About 0.1ml of both nodule and rhizosphere soil solutions were spread on petriplates having YEMA media and spread well with the help off spreader. The plates were incubated at 28 ºC ± 2º for 2 days. Isolated colonies of *Rhizobium* isolates were transferred on YEMA slants and were stored in refrigerator at 4 ºC for further studies. (Cappucino *et al*., 2007) [14]

Morphological Characterization
The colony characteristics of the isolates size, shape, colour, texture and elevation on YEMA was recorded after 2days of incubation at 28º±2º (Shaikhul Islam., 2016) [49]. Gram staining was carried out as described by (Vincent Humphery, 1970) [63].

Biochemical Characterization
Isolates were biochemical characterized for various tests such as IMViC, starch hydrolysis, sugar fermentation, gelatin hydrolysis, catalase, oxidase, urease test and bromo thymol blue was performed. (Bergeys's *et al*., 1994) [11].

Physiological Characterization
Isolates were assessed for their physiological characteristics temperature, P^H, salt concentration, carbohydrate utilization test and amino acid utilization test was performed.

Effect of Temperature on *Rhizobium*
To study the growth of the isolates at different incubation temperature the isolates were streaked on YEMA and incubated at 15 ºC, 20 ºC, 25 ºC, 30 ºC, 35 ºC, 40 ºC and 45 ºC as described by (Erana Kebede *et al*., 2021) [19]. To study comparison among the isolates at different temperatures continuous growth at 28 ºC ±2 ºC was used as control. Results were recorded as good growth, moderate growth, poor growth and no growth. (Alexandre and Oliveira, 2011) [3]

Effect of P^H
Growth of *Rhizobium* on YEMA with various P^H *viz*., 5, 6, 7, 8, 9, 10 was studied. The P^H was adjusted using 0.1 N Hcl and 1N NaoH.

Effect of NaCl
The capacity of the isolates to grow at various salt concentrations was assessed by inoculating each isolate on YEMA added with Nacl 0.1%, 0.4%, 0.8%, 1%, 2%, 3%, 4%, 5%, 6%, 7% concentration. (Lupwayi and Haque., 1994) [32].

Effect of Carbon sources
Utilization of carbohydrates by the isolates was tested by supplementing the media with different sugars *viz*., dextrose, xylose, sucrose, lactose, maltose, fructose, starch and cellulose (Somasegaran and Hoben, 1991) [69].

Effect of Nitrogen sources
Amino acid *viz*., tryptophan, asparticacid, arginine, lysine, tyrosine, methionine and glutamate were added to the media at a concentration of 0.5gm/lt in order to determine the ability of the isolates to utilize amino acid as nitrogen source. (Erana Kebede *et al*., 2021) [19].

Screening of *Rhizobium* Isolates for Plant Growth Promoting Traits
Phosphate Solubilization
Phosphate solubilization ability was recorded by spot inoculation of the isolates on Sperber medium. The plates were incubated at 28 ºc. The presence of clear zone around the colony of the isolate indicate positive result. The diameter of zone of clearance surrounding the bacterial colony as well as the diameter of the colony was measured after 48 hours. (Rahim Nosrati *et al*., 2014) [44].

IAA production
A colorimeter technique was performed Van Urk Salkowski reagent using the salkowski's method (Ehmann 1977) [70] to determine the amount of IAA produced by each isolate. The isolates were grown on YEM Broth (Hi media, India) supplemented with tryptophan as precursor at the rate of 1mM and incubated at 28 ºc for 4 days. The broth was centrifuged after incubation supernatant was retained and 1ml was mixed with 2ml of Salkowski's reagent and kept in dark. The optical density was recorded at 530nm using spectrophotometer. (B Mohite 2013) [9].

Ammonia Production
To detect the production of ammonia, *Rhizobium* isolates were inoculated in peptone water and incubated at 28 ºC for 72hours. After incubation 0.5ml of Nessler's reagent was added to each tube the development of brown colour indicated production of ammonia. (Sirasagikar Reshma N. *et al*. 2023) [50].

HCN Production
HCN production was observed by using Kings B broth supplemented with 4.4g/lt of glycine *Rhizobium* isolates were inoculated into the broth Whatmann filter paper no.1 (9mm in diameter) soaked in 2% sodium carbonate in 0.5% picric acid solution was fixed underside of the test tube containing broth the mouth of the test was sealed using parafilm and incubated at 28 ºC for 7 days. Formation of deep yellow colour to dark brown confirms production of HCN. (Bakker *et al*., 1987 and Monika., 2017) [10, 39].

Siderophore Production
All isolates were detected for siderophore production by CAS (Chrome Azurol S) assay. The isolates were spot inoculated on CAS plates and incubated at 28 ºC for 5 -7 days. Siderophore presence is indicated by the decolourization of the blue coloured dye into yellow halozone around the colonies. (Brain C. Louden *et al*., 2011) [13].

Nitrate Reduction Test
Detection of nitrate reduction was done using nitrate broth. Nitrate broth was prepared and inoculated with actively growing culture and incubated for 2 to 3 days at 28ºc. After incubation period 5 drops of sulphanilic acid and alpha-naphthol amine dissolved in acetic acid was added to the broth. Development of red colour indicates nitrate reduction activity. (Sahar Alipour Kaif *et al*., 2021) [47].

***In vitro* Nitrogen Fixation**
In vitro nitrogen fixation by the *Rhizobium* isolates was determined by Kjeldahl method (Humpries 1965) [26]

Results and Discussion
Among 83 *Rhizobium* isolates isolated from the root nodules of Pigeon pea plant collected from the fields nearby Raichur Thermal Power Station (RTPS), based on their growth characteristics 10 isolates were selected. (Monika *et al*., 2017) [39] Morphological characteristics of selected isolates was studied. The colonies of the *Rhizobium* isolates produced white, milky, circular, gummy, convex, raised, translucent and smooth surface colonies. (Plate-1) The Gram staining result showed them to be Gram negative rods. (Table 1)

(Kumar *et al*., 2017 and Shaihul Islam., 2016) [61, 49] also observed same morphological features for *Rhizobium*.

Biochemical Characterization

The biochemical results of selected isolates is presented in Table 1. Isolates BUDR09, BUDR12, BUDR13, BUDR20, BUDR21, BUDR26, BUDR27, BUDR38, BUDR40, BUDR42 was streaked on YEMA supplemented with Bromo Thymol blue (BTB) after an incubation period of 2 days the growth of the isolates BUDR12, BUDR13, BUDR26, BUDR27 AND BUDR44 was visible the YEMA media turned from blue to yellow representing them as acid producers BUDR09, BUDR20, BUDR21, BUDR38 and BUDR40 did not turned the media from blue to yellow. Similar results were observed by (Zeenat Wadhwa *et al*., 2017) [67]. IMViC test was conducted on *Rhzobium* isolates BUDR13, BUDR21, BUDR26 and BUDR27 were positive for indole test, whereas BUDR09, BUDR12, BUDR20, BUDR30, BUDR40 and BUDR44 were indole negative. BUDR09, BUDR13, BUDR26, BUDR27, BUDR38, BUDR40 were positive for methyl red test whereas BUDR12, BUDR20, BUDR21 and BUD44 were negative for methyl red test. All the 10 isolates *viz*., BUDR09, BUDR12, BUDR13, BUDR20, BUDR21, BUDR26, BUDR27, BUDR38, BUDR40 and BUDR44 were negative for VP test. BUDR09, BUDR13, BUDR26, BUDR27, BUD38, BUD40 and BUDR40 were positive for citrate utilization, whereas BUDR12, BUDR20, and BUDR21 were negative. Starch hydrolysis test was performed on all the isolates, BUDR12, BUDR13, BUDR27, BUDR40 showed positive for starch hydrolysis but BUDR09, BUDR20, BUDR21, BUDR26, BUDR38, BUDR44 were negative. Isolates BUDR12, BUDR20, BUDR38 hydrolysed gelatin whereas BUDR09, BUDR13, BUDR21, BUD26, BUDR27, BUDR40 and BUDR44 failed to hydrolyse gelatin. Catalase, oxidase and urease test was also performed on all the isolates. Isolates BUDR09, BUDR12, BUDR13, BUDR20, BUDR21, BUDR26, BUDR27, BUDR38, BUDR40 and BUDR44 were positive for catalase and oxidase test. Isolates BUDR12, BUDR13, BUDR20, BUDR21, BUDR26, BUDR27 and BUDR44 were positive for urease test but isolates BUDR09 and BUDR40 were negative. (Table 2) (Meenakshi Dhiman *et al*., 2019) [35] also reported the same.

Sugar Fermentation Test

Carbohydrates fermentation test was carried on all the *Rhizobium* isolates. The isolates BUDR09 utilised fructose, mannitol, sucrose, maltose and glucose producing acid and gas but failed to ferment lactose. BUDR12, utilized mannitol, sucrose, maltose glucose and lactose producing acid and gas but fructose was fermented by producing only acid not gas. BUDR13, utilized fructose, mannitol, sucrose, maltose, glucose and lactose producing acid and gas BUDR20 utilized fructose, mannitol, sucrose, maltose and glucose producing acid and gas but failed to ferment lactose., BUDR21, utilized fructose, mannitol, sucrose, maltose and glucose producing acid and gas but failed to ferment lactose., BUDR26, utilised fructose, mannitol, sucrose, maltose, glucose and lactose producing acid and gas BUDR27, utilised fructose, mannitol, sucrose, maltose, glucose and lactose producing acid and gas, BUDR38, utilised fructose, mannitol, sucrose, maltose and glucose producing acid and gas but failed to ferment lactose. The isolate BUDR40 utilised fructose, mannitol, sucrose, maltose, glucose and lactose producing acid and gas and isolate BUDR44 utilised fructose, mannitol, sucrose, maltose and glucose producing acid and gas but failed to ferment lactose (Table 3) Similar result was observed by (Deora and Singhal 2010.) [22] Utilization of carbon sources by bacterial isolates might indicate their metabolic and ecological diversity suggesting their use as biofertiliser /biocontrol agent (Deng *et al*., 2011; Fitriyah *et al*., 2013; Mantilla- Afanador *et al*. 2017) [17, 71, 34].

Physiological Characterization

Effect of temperature: The *Rhizobium* efficient strain were inoculated on YEMA broth and incubated at various temperature range *viz*., 15 ºC, 25 ºC, 30 ºC, 35 ºC, 40 ºC and 45 ºC. Isolate BUDR13 showed poor growth at 15 ºC, good growth was recorded at temperature 25 ºC, 30 ºC and 35 ºC whereas at 40 ºC and 45 ºC no growth was observed. Isolate BUDR27 also showed less growth at 15 ºC, good growth was recorded at 25 ºC, 30 ºC and 35 ºC, but at 40 ºC and 45 ºC no growth was observed. (Table 4) Low temperature decreases growth rate, enzyme activity and alters cell composition. The high temperature is reported to alter the permeability of the membrane and leading to denaturation of enzymes/proteins causing the death and/ or poor growth of the rhizobia (Bhargava *et al*., 2016, Hungria and Vargas 2000 and Florentine *et al*., 2010) [12, 72, 21]. The suitable temperature for the growth of rhizobia has been reported to be between 25 ºC and 30 ºC (Zhang *et al*., 1995) [73] which matched with our results.

Effect of P^H: P^H is one of the essential growth factor for the bacteria to grow. *Rhizobium* Isolate BUDR13 showed good growth on media with P^H7 and 8, moderate growth was observed at P^H6 and poor growth was recorded at P^H5, P^H 9and P^H 10. Isolate BUDR27 grew good at P^H5, P^H6 and P^H 7, moderate growth was observed at P^H8 and no growth was observed at P^H9 and P^H10. (Table 7) Low P^H can cause changes in the bacterial cell membrane and walls. This can lead to membrane permeability changes, disrupting essential cellular process and structures such as ion exchange, metabolism and protein synthesis whereas at high P^H the hydroxide ion (OH^-) concentration increases, making the environment more basic or alkaline hence the cell membrane of bacteria can become damaged and lose its selective permeability, leading to loss of cellular contents and ultimately, cell death. (Asnake Beshah and Fassil Aseefa., 2019) [6] reported *Rhizobium* showed growth on wide range of P^H *viz*., P^H4 to P^H9 (Kaur *et al*., 2012) [29] observed that the ideal rhizobial growth on media with neutral P^H which is similar with our result. (Florentino *et al*., 2010) [21]; reported Rhizobial isolates that showed growth at P^H5 are often very important candidates as inoculant and for biofertilizer production in the acidic soils of cowpea producing areas to realize higher yield. (Erana Kebede *et al*., 2021) [19] stated that rhizobial isolates of few leguminous plant could grow at P^H values ranging between ph5.0 and P^H11.0 (Mainak Bhattcharjee 2018) [33] reported highest growth of rhizobial isolates was obtained P^H 7.0. P^H around neutral is ideal for the uptake of a proper amount of nutrients and allows optimal growth of the rhizobia isolates. (Bhargava *et al*., 2016) The growth was declined at higher and lower P^H *viz*., P^H11.0 and P^H4.0 (Del papa *et al*., 1999) [16] which matched with our results.

Effect of NaCl Concentration: The potential strains of *Rhizobium* were tested in different concentration of salt. The efficient isolates showed great changes in their growth on the media. The suitable salt concentration for the good growth of isolate BUDR13 is 0.1%, 0.4%, 0.8%, 1%, 2%, 3%, of NaCl, moderate growth was seen at 4% and 5% and no growth was observed at 6% and 7% of NaCl. Whereas isolate BUDR27 showed good growth at 0.1%, 0.4%, 0.8%, 1%, 2%, 3% of NaCl, less growth was observed on 4% and 5% whereas on 6% and 7% NaCl there was no growth (Table 8). Salinity is considered a limiting factor in nodulation and nitrogen fixation in legume *Rhizobium* associations, which can adversely affect the yield of legume crop. Salinity decreases rhizobial growth due to osmotic stress and higher concentration of toxic ions. 1% concentration of NaCl is suitable for *Rhizobium* growth. Higher concentration of NaCl inhibits its growth. (Mohammed RM *et al*., 2015, Zahran 1999 and Mainak Bhattachargee *et al*., 2018) [40, 66, 33] As well as (Nushair *et al*., 2017 and Zeenat Wadhwa., 2017) [74, 67] reported that *Rhizobium* were able to grow at 1%. NaCl but incapable to tolerate and grow at higher concentration of NaCl. (Table 8).

Effect of Carbon Sources

Isolate BUDR13 utilized various sugars as carbon source. Abundunt growth of the isolate on the media containing dextrose, fructose, xylose, and maltose was recorded as good growth whereas with lactose, sucrose, cellulose showed moderate growth and with starch poor growth was observed. Isolate BUDR27 showed good growth on media supplemented with dextrose, fructose, xylose, moderate growth was observed with lactose, maltose, and sucrose, poor growth was recorded with starch and cellulose (Table 9) (Asnake Beshah *et al*., 2019) [6] also reported the same which matched with our results.

Effect of nitrogen sources: Efficient *Rhizobium* isolate BUDR13 utilised tryptophan, aspartic acid, aspargine, lysine, tyrosine, glutamate when grown on the media supplemented with these different nitrogen sources. The results are recorded as good growth, whereas with methionine the isolate showed moderate growth. Isolate BUDR27 were able to catabolize amino acids tryptophan, aspartic acid, arginine, lysine, tyrosine, glutamate with good growth on media, with methionine the isolate showed moderate growth, (Table 10) which was also reported by Erana Kebede *et al*., 2021 [19].

Screening of *Rhizobium* Isolates for PGPR Activity

All the *Rhizobium* isolates were screened for their PGPR activity. In present study, various PGP activity tests were carried out to select efficient strains of *Rhizobium*. Table –4 and Fig-1, depicts qualitative analysis of *Rhizobium* isolates (%) that indicates PGP activity. Phosphate solubilizing microorganisms have been shown to improve soil borne plant pathogen (Vassilev *et al*., 2006: Wani *et al*., 2007) [60, 64] in our study 70% isolates *viz*., BUDR09, BUDR12, BUDR13, BUDR26, BUDR27, BUDR40, BUDR44 were positive for phosphate solubilization, 30% isolates failed to solubilize phosphate, BUDR20, BUDR21 and BUDR38 showed negative result for phosphate solubilization. Among all the isolate BUDR13 shows the highest P solubilization of 22.2mm followed by BUDR27 with a 21mm solubilization zone and isolate BUDR44 showed least P solubilization of 14.6mm (Fig-2). Indole -3- acetic acid is an important phytohormone which enhances cell elongation, root length, shoot growth, cell division and differentiation in plants. (Monika *et al*., 2017) [39] Bacteria uses this phytohormone to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defence mechanisms (Spaepen *et al*., 2007) [54] 100% isolates *viz*., BUDR09, BUDR12, BUDR13, BUDR20, BUDR21, BUDR26, BUDR27, BUDR38, BUDR40 and BUDR44 showed positive result for IAA production. The quantitative result of IAA production is represented in Table- 5, the highest IAA production was observed with the isolate BUDR13 with 19.46µg/ml^{-1} followed by BUDR27 with 17.98µg/ml^{-1} (Fig-4) whereas the isolate BUDR20 produced least of IAA with 6.62µg/ml^{-1}. For comparison a reference strain of *Rhizobium* was collected from department of microbiology, agriculture university, Raichur. Ammonia is one of the important traits of PGPR that may indirectly influence plant growth, (Sakthivel. U and Karthikeyan., 2012) [48], 60% isolates BUDR13, BUDR20, BUDR26, BUDR27, BUDR40 and BUDR44 were positive for ammonia production whereas 40% BUDR09, BUDR12, BUDR21 and BUDR38 showed negative result. HCN production by the *Rhizobium* isolates also influences plant growth and inhibit plant disease development thus strengthening the host's disease resistance mechanism (Monika *et al*., 2016) [10] 50% isolates *viz*., BUDR09, BUDR13, BUDR21, BUDR27, BUDR40 showed positive for HCN production and 50% isolates BUDR12, BUDR20, BUDR26, BUDR38 and BUDR44 were negative for HCN production. Siderophore chelates iron and other metals suppresses diseases by conferring a competative advantages to biocontrol agents for the limited supply of essential trace minerals in natural habitats (Henkals, 1997) [27] Siderophore production by *Rhizobium* strains has been considered as a potential way to improve nodulation and nitrogen fixation in iron deficiency conditions (Arora *et al*., 2001) [5] in our study 40% isolates *viz*., BUDR12, BUDR13, BUDR21 and BUDR27 were positive for Siderophore production and remaining 60% *viz*., BUDR09, BUDR20, BUDR26, BUDR38, BUDR40 and BUDR44 were non siderophore producers. 100% isolates *viz*., BUDR09, BUDR12, BUDR13, BUDR20, BUDR21, BUDR26, BUDR27, BUDR38, BUDR40 and BUDR44 showed positive results (Plate -2) for nitrate reduction tests.

***In vitro* Nitrogen Fixation by *Rhizobium* isolates**

All the *Rhizobium* isolates fixed N_2 in the YEMA broth medium, ranged from 5.22 to 16.27 mg/g of mannitol. (Table-V) Isolate BUDR13 fixed highest amount of nitrogen *viz*., 16.27 mg/g followed by the isolate BUDR27 14.56 mg/g of mannitol.(Fig-3) Whereas the isolate BUDR12 has fixed lowest amount of nitrogen *viz*., 5.22mg/g of mannitol.

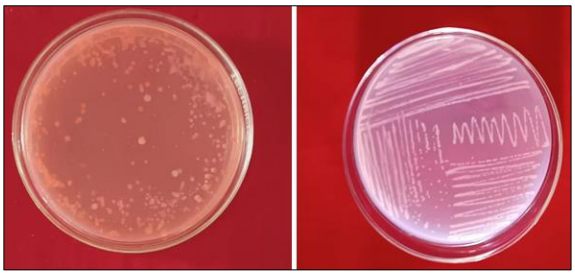


Plate 1: Colonies of *Rhizobium* on YEMA

Table 1: Morphological and Microscopic Characterization of *Rhizobium* Isolates

Isolates	Colony Characteristics	Gram's Reaction	Cell Shape
BUDR09	Small circular, white, gummy, convex, translucent	-	Rods
BUDR12	Small, Circular, white, gummy, convex, translucent	-	Rods
BUDR13	Medium, circular, milky, gummy, raised, translucent	-	Rods
BUDR20	Medium, circular, white, gummy, raised, translucent	-	Rods
BUDR21	Medium, circular, white, gummy, convex, translucent	-	Rods
BUDR26	Medium, circular, white, gummy, convex, translucent	-	Rods
BUDR27	Small, circular, milky, gummy, convex, translucent	-	Rods
BUDR38	Small, circular, white, gummy, raised, translucent	-	Rods
BUDR40	Small, circular, white, gummy, convex, translucent	-	Rods
BUDR44	Small, circular, white, gummy, convex, translucent	-	Rods

Table 2: Biochemical Characterization of *Rhizobium* Isolates

Isolates	BTB	I	M	V	C	Starch Hydrolysis	Gelatin Hydrolysis	Catalase	Oxidase	Urease
BUDR09	-	-	+	-	+	-	-	+	+	-
BUDR12	+	-	_	-	-	+	+	+	+	+
BUDR13	+	+	+	-	+	+	-	+	+	+
BUDR20	-	-	_	-	-	-	+	+	+	+
BUDR21	-	+	_	-	-	-	-	+	+	+
BUDR26	+	+	+	-	+	-	-	+	+	+
BUDR27	+	+	+	-	+	+	-	+	+	+
BUDR30	-	-	+	-	-	-	+	+	+	+
BUDR40	-	-	+	-	+	+	-	+	+	-
BUDR44	+	-	-	-	-	-	-	+	+	+

BTB: Bromo Thymol Blue, I: Indole, M: Methyl Red. Vp: Voges Proskauer, C: Citrate Utilization, (+) Positive (-) Negative

Table 3: Sugar Fermentation Test on *Rhizobium* Isolates

Isolates	Fructose		Mannitol		Sucrose		Maltose		Glucose		Lactose	
	A	G	A	G	A	G	A	G	A	G	A	G
BUDR09	+	+	+	+	+	+	+	+	+	+	-	-
BUDR12	+	-	+	+	+	+	+	+	+	+	+	+
BUDR13	+	+	+	+	+	+	+	+	+	+	+	+
BUDR20	+	+	+	+	+	+	+	+	+	+	-	-
BUDR21	+	+	+	+	+	+	+	+	+	+	-	-
BUDR26	+	+	+	+	+	+	+	+	+	+	+	+
BUDR27	+	+	+	+	+	+	+	+	+	+	+	+
BUDR38	+	+	+	+	+	+	+	+	+	+	-	-
BUDR40	+	+	+	+	+	+	+	+	+	+	+	+
BUDR44	+	+	+	+	+	+	+	+	+	+	-	-

A: Acid, G: Gas, (+): Positive, (-): Negative

Table 4: Screening of Efficient Isolates for PGPR Traits

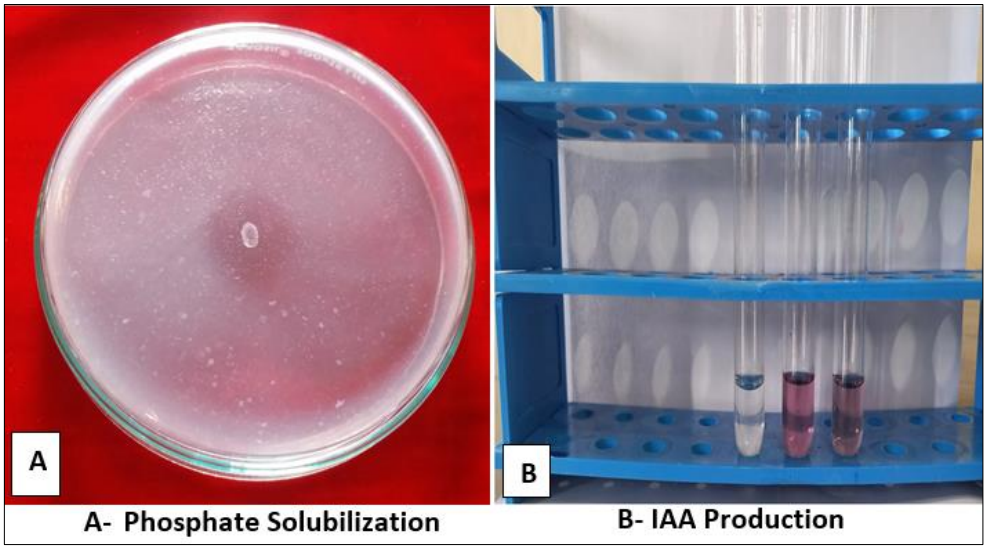
Isolates	Phosphate Solubilisation	IAA Production	Ammonia Production	HCN Production	Siderophore Production	Nitrate Reduction
BUDR09	+	+	-	+	-	+
BUDR12	+	+	-	-	+	+
BUDR13	+	+	+	+	+	+
BUDR20	-	+	+	-	-	+
BUDR21	-	+	-	+	+	+
BUDR26	+	+	+	-	-	+
BUDR27	+	+	+	+	+	+
BUDR38	-	+	-	-	-	+
BUDR40	+	+	+	+	-	+
BUDR44	+	+	+	-	-	+

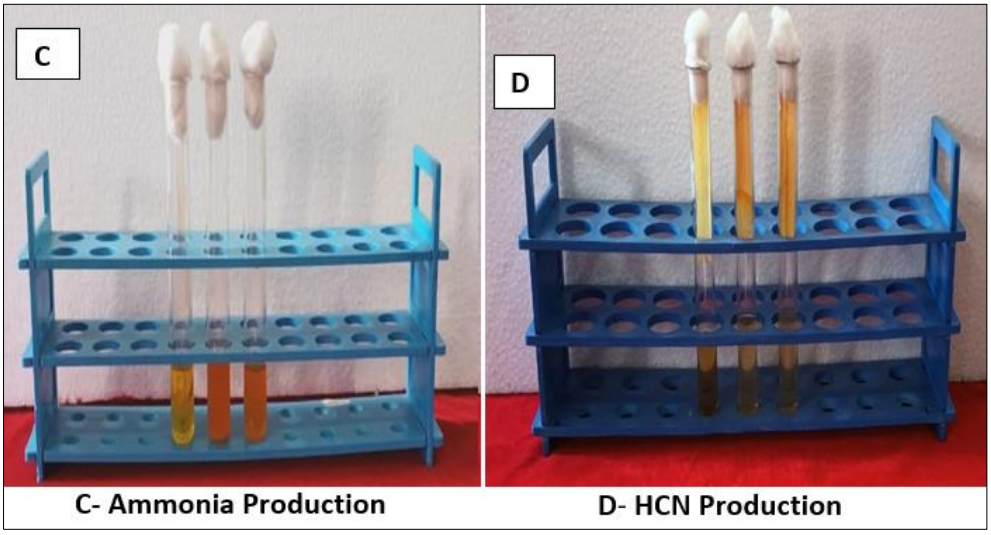
(+): Positive and (-): Negative

Table 5: PGPR and Nitrogen Fixation Activity of *Rhizobium* isolates

Isolates	Phosphate solubilization zone diameter	*In vitro* $N_{2\ fixation}$(mg N_2 fixed g^{-1}) of mannitol	IAA production (µg/ml^{-1})
BUDRO9	19mm	12.03	10.60
BUDR12	16mm	5.22	7.01
BUDR13	22.2mm	16.27	19.46
BUDR20	17.3mm	9.12	6.62
BUDR21	15mm	9.32	8.04
BUDR26	119.5mm	11.06	12.04

BUDR27	21mm	14.56	17.98
BUDR38	20mm	6.44	7.52
BUDR40	17mm	8.13	10.30
BUDR44	14.6mm	10.33	11.06
Ref strain	20.34	14.16	17.90
S.Em±	0.2082	0.1861	0.2371
CD 1%	0.8377	0.7488	0.9540





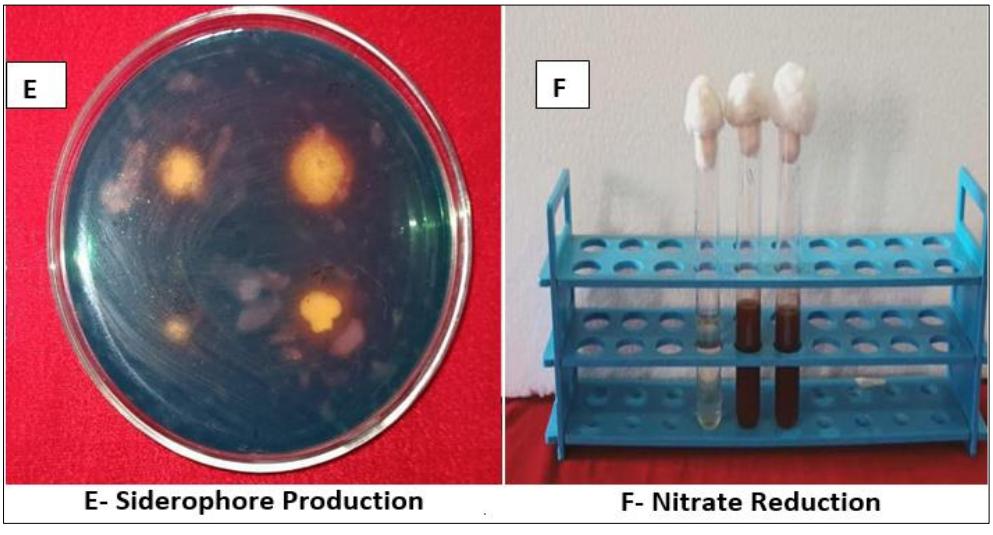


Plate 2: Screening of Efficient Isolates for PGPR Traits

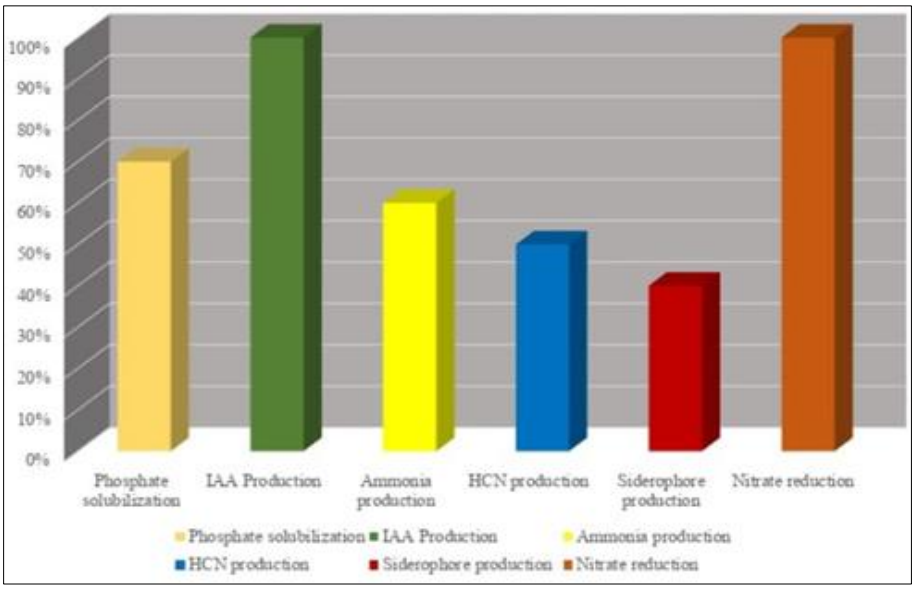


Fig 1: Screening of Efficient Isolates for PGPR Traits

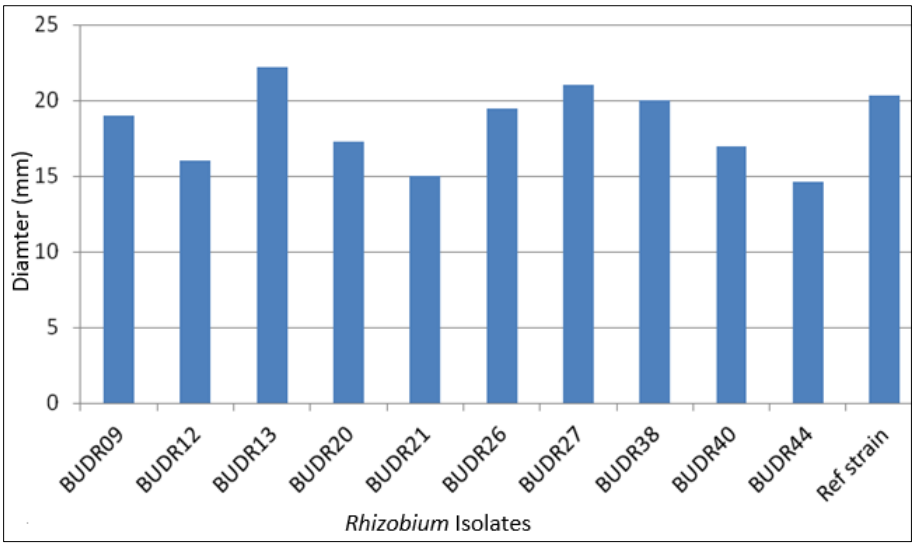


Fig 2: Screening of *Rhizobium* isolates for P *solubilization*

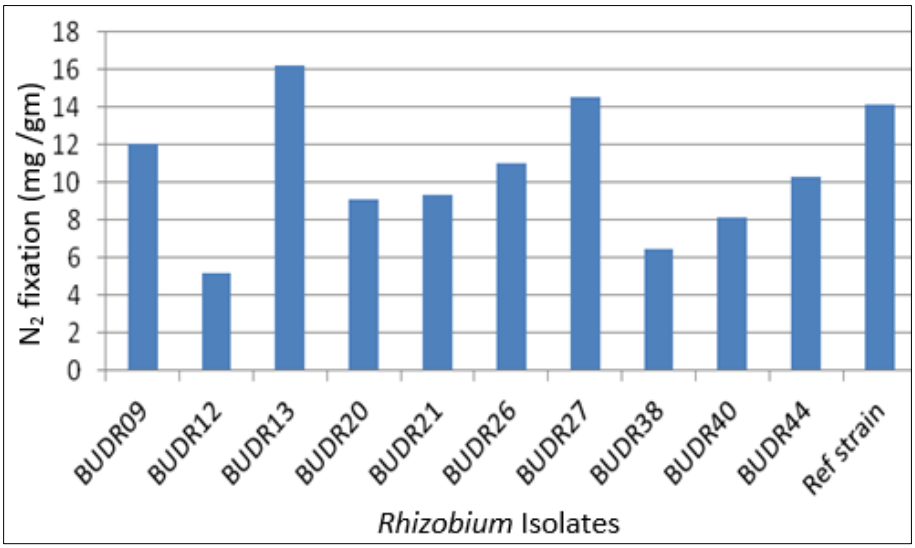


Fig 3: Nitrogen fixed by *Rhizobium* isolates

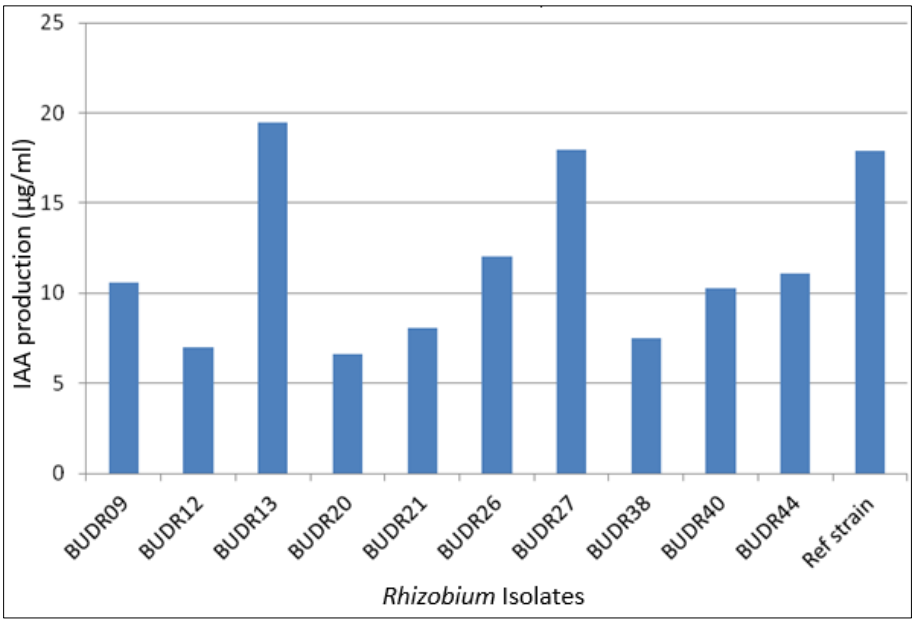


Fig 4: Screening of *Rhizobium* isolates, IAA production

Table 6: Effect of Temperature

Isolates	15 ºC	25 ºC	30 ºC	35 ºC	40 ºC	45 ºC
BUDR13	+	+++	+++	+++	-	-
BUDR27	+	+++	+++	+++	-	-

(+++) Good Growth, (++) Moderate Growth, (+) Poor Growth and (-) No Growth

Table 7: Effect of P^H

Isolates	PH5	PH6	PH7	PH8	PH9	PH10
BUDR13	+	++	+++	+++	-	-
BUDR27	+++	+++	+++	++	-	-

(+++) Good Growth, (++) Moderate Growth, (+) Less Growth (–) No Growth

Table 8: Effect of NaCl

Isolates	0.1%	0.4%	0.8%	1%	2%	3%	4%	5%	6%	7%
BUDR13	+++	+++	+++	+++	+++	+++	++	++	-	-
BUDR27	+++	+++	+++	+++	+++	+++	+	+	-	-

(+++) Good Growth, (++) Moderate Growth, (+) Poor Growth (–) No Growth

Table 9: Effect of Carbon Sources

Isolates	Dextrose	Fructose	Xylose	Lactose	Maltose	Sucrose	Starch	Cellulose
BUDR13	+++	+++	+++	++	+++	++	+	++
BUDR27	+++	+++	+++	++	++	++	+	+

(+++) Good Growth, (++) Moderate Growth, (+) Poor Growth (–) No Growth

Table 10: Effect of Nitrogen Sources

Isolates	Tryptophan	Aspartic acid	Arginine	Lysine	Tyrosine	Methionine	Glutamate
BUDR13	+++	+++	+++	+++	+++	++	+++
BUDR27	+++	+++	+++	+++	+++	++	+++

(+++) Good Growth, (++) Moderate Growth, (+) Poor Growth (–) No Growth

Conclusion

In the present study 83 *Rhizobium* isolates were isolated from root nodule of Pigeon pea plant. Isolate BUDR13 and BUDR27 were efficient isolates since these were efficient in solubilizing of phosphate, nitrogen fixing efficiency higher production of IAA and they are able to reduce soil borne pathogens as well as produced ammonia, HCN, siderophore, which are important plant growth promoting traits. As well as these strains grew well when amended with 10% flyash. Flyash plays an important role for the growth and yield of leguminous plants. Flyash is a rich source of all essential macro and micro nutrients for the plant growth. (Meenakshi Sharma *et al*., 2018) [36]. Hence BUDR13 and BUDR27 can be used as bioinoculant for leguminous plants which helps in maximum crop yield and protect the crop plant from the hazardous effects of chemical fertilizers used in the agricultural fields for the quick yield of crop. The strain survives at higher temperature, and temperature fly ash has no impact on the survivability of the isolates under varied climatic resilience it would have been the promising isolate for production of bioinoculants for leguminous crops of this Kalyan Karnataka (KK) region for enhancing nutritional mineralization under fragile ecosystem.

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