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Isolation, screening and Biochemical identification of plant growth promoting Rhizobacteria of *Lablab purpureus* (L.) Sweet

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Abstract

In the current study, rhizospheric soil and nodules from Indian bean are used to isolate, a total of twelve colonies of distinct rhizobacteria on YEMA media, through serial dilution method. Soil samples were collected from nine randomly selected healthy Dolichos bean plants containing root nodules and root-adhered soil. A total of twelve isolated bacteria were screened *in vitro* for different plant growth promotion activities *i.e.* phosphate solublization, IAA production, siderophore production, ammonia production and biochemical testing. In the present work out of twelve bacterial isolates six was positive for phosphate solublization. IAA production and siderophore production was shown by eleven bacterial isolates. All isolates were positive for ammonia production, starch hydrolysis, citrate and catalase test and further identified on the basis of colony morphology, Gram staining and biochemical tests. These isolates were identified as *Escherichia. coli*, *Bacillus* sp. As PGPR are environmental friendly and offer sustainable approach to increase production of crops and health. Therefore, these isolates can be utilized for biofertilizer formulation under local agro-climatic conditions.

Key words : PGPR, *Bacillus* sp., Rhizosphere, Phosphate solublization, siderophore, Ammonia production.

The Rhizosphere, a limited soil region, is significantly affected by the interaction between the soil microbiome and the secretions of plant roots. These root secretions consist of primary metabolites such as sugars, organic acids, and amino acids, which play a crucial role in shaping the dynamics of the surrounding soil environment (Walker *et al.,* 2011). The growth and yield of plants directly and indirectly elevated by soil

microbiome, referred as Plant Growth Promoting Rhizobacteria (PGPR). There are several direct mechanisms through which PGPR are known to enhance plant growth are nutrient solubilization, atmospheric nitrogen fixation; production of siderophore for iron uptake; phosphorous solubilization, modulation of plant growth hormones, such as synthesis of the enzyme ACC deaminase, which cleaves ethylene hormone, that plays a crucial role in plant's response to stress (Bal *et al.,* 2013), production of indole-3-acetic acid (IAA) for root development and the production of plant regulators involved in various cellular processes and developmental pathways. These regulators include gibberellins, cytokinins, abscisic acid, salicylic acid, brassinosteroids and jasmonates. The indirect mechanism through which PGPR are known to regulate the growth of plant by inhibiting the functioning of one or more plant pathogenic organisms both bacteria and fungi; cell wall degrading enzymes; antibiotics; competition; quoram quenching and induced systemic resistance[10]. However, all of these responses cannot elicited only by single microorganism, so instead using a single strain, the practice of plant-growth-promoting consortia, is of current research interest[5].

Amid growing international dread regarding food and environmental quality, the utilization of PGPR has emerged as a pivotal matter in mitigating the assurance on chemical inputs within the agricultural sector. PGPR have been implemented to diverse crops to enhance crop yield, seed emergence, growth and even have the potential for commercialization. According to Ramirez-Carino *et al.,* (2023) treated tomatillo seedlings with isolated bacterium identified as *Atlantibacter calcoaceticus*, *Priestia megaterium* have higher root length (>11%) and leaf weight (>349%) than the control. *Pseudomonas fluorescens* isolated from roots of gramineous plants have been recorded to colonize tomato plants and increased its number of fruit, flower as well as a higher overall fruit weight. Recently, PGPR are the growing interest in agriculture as growth promoting and biological control agents due to their efficacy but there is very trivial data regarding the practice of PGPR as biofertlizer.

Currently, the market for PGPR-based biofertilizer accounts for approximately 5% of the total market share occupied by chemical fertilizers in agricultural practices. Keeping all of this in view the current study was taken for testing the competency of PGPR for Auxin production, phosphate solublization and siderophore formation after biochemical testing of isolated bacteria from the rhizosphere with intact root nodules of *Lablab purpureus* (Dolichos bean). As it is primitive legume crop widely cultivated throughout the world for its multiple purposes including human consumption as pulses or vegetables, animal feed and as forage for livestock. Dolichos beans are rich source of many nutrients like vitamin A, vitamin B1, vitamin C and minerals like protein, folate, copper, phosphorus, potassium, iron, manganese, molybdenum and dietary fibre[1].

Seed procurement and experimental design:

Seeds of Dolichos bean were procured from IARI, New Delhi and sown at Botanical garden of Raja Balwant Singh college of Dr. Bhimrao Ambedkar university Agra. A plot dimension was 4.88m×3.96m size, in which 9 row and 6 column was created and 6 seeds

were sown in the distance of 2M in each row in month of July, proper watering of plants and weed removal actively were done for four months till flowering occur in the month of November. After four months, soil samples were collected in zipper plastic bags from nine randomly selected healthy Dolichos bean plants containing root nodules and root-adhered soil till 20cm depth. Collected soil were placed in icebox (4°C) and transported to the laboratory of Raja Balwant Singh College, Agra till further studies.

Bacterial isolation :

After 24 hr incubation rhizospheric soil and root nodules bacteria were isolated from the samples. Nodules were washed under running tap water, before dipping into mercuric chloride $HgCl_2$ solution (5 minutes) and followed by ten times wash with distilled water to remove traces of adhered $HgCl_2$ on the nodule surface. Sterilized nodules were transferred into sterile culture test tube containing 5ml of sterilized distilled water and crushed thoroughly. one full loop of macerate was streaked on nutrient agar plate and incubated at a temperature of 28°C for a period of 48-72 hr.[9].

One gram soil sample was serially diluted for isolating the bacteria from soil sample, by adding it to 9ml of distilled water in test tubes to achieve a total volume of 10ml, an initial dilution of 10^{-1} was prepared. Subsequently, 1ml of the mixture was transferred from the 10^{-1} dilution and added to a second tube, resulting in a total dilution factor of 10^{-2} and this process was repeated iteratively upto a dilution factor 10^{-6}. Then an aliquot of 10^{-3} dilution was spread on the nutrient agar plates and incubated (28°C, 3 days) for appearing the colonies of bacteria. Twenty-seven isolated colonies were picked up from nutrient agar plates and re-streaked on fresh yeast extract-mannitol-mineral salts agar plate (YEMA) medium, only twelve colonies survived. These colonies were used for analysis of morphological characters, screening, biochemical tests and subsequent sub-culturing[6].

Biochemical tests :

Catalase test : Qualitative catalase activity of bacterial strain was tested following. A loop of 18 to 24 hr old isolated bacterial culture were smeared on clean, dry glass slide and a drop of 3% H_2O_2 mixed with sterile tooth pick, evolution of oxygen was observed by effervescences which shows the positive catalase activity[8].

Citrate test : The bacterial isolates were qualitatively tested for citrate as source of energy by Simmons's citrate agar having Bromothymol blue indicator. The isolates were picked from the centre of a colony and streaked onto slanted media using a back-and- forth motion in test tubes, incubated at a temperature of 35-37°C aerobically and observed for colour change from green to blue along the slant for citrate utilization.

Starch Hydrolysis : Starch hydrolysis ability of isolates was tested by streaking them on a starch plate in the form of a line across the plate and flooding with an iodine solution. Appearance of clear zone around the line of bacterial colony after 48 hr indicated the positive starch hydrolysis[4].

Table-1. Bacterial isolates with various plant growth-promoting properties

Bacterial isolates code	Catalase test	Citrate test	Starch hydrolysis test	Ammonia production	IAA production	Phosphate solubilisation	Siderophore producing bacteria	Bacteria identification
Plate1 Cream (P1C)	+	+	+	+	+	-	+	*Escherichia coli*
Plate2 Green/white (P2G/W)	+	+	+	+	+	+	+	
Plate3 Cream (P3C)	+	+	+	+	+	-	+	*Escherichia coli*
Plate4 White (P4W)	+	+	+	+	+	+	+	*Bacillus. cereus*
Plate5 Cream (P5C)	+	+	+	+	+	+	+	
Plate6 Cream (P6C)	+	+	+	+	+	+	+	*Bacillus cereus*
Plate6 White (P6W)	+	+	+	+	+	+	+	*Bacillus subtilis*
Plate7 White (P7W)	+	+	+	+	+	-	+	*Escherichia coli*
Plate8 Yellow (P8Y)	+	+	+	+	+	+	+	*Bacillus cereus*
Plate9 White (P9W)	+	+	+	+	-	-	-	
Plate9 Green/white (P9G/W)	+	+	+	+	+	-	+	*Bacillus cereus*
Plate9 Pink/ white (P9P/W)	+	+	+	+	+	-	+	*Escherichia coli*

Ammonia production test : The isolated bacteria were tested qualitatively for ammonia production in peptone water. The test tubes poured with 10ml of peptone water with inoculums of freshly grown cultures and incubated (48-72 hr, 28-30°C) following addition of 0.5 ml Nesseles's reagent. The development of brown to yellow colour indicated ammonia production.

Screening of isolates :

IAA production : Qualitatively assayed for IAA production was done by plate assay. In brief tryptic soy agar (5.0g NaCl, l^{-1}; 5.0g peptic digest of soybean meal; 15.0g agar) with 100µg/ml L-tryptophan) plates were prepared and dividing into two equal halves. Isolates were streaked with sterile loop on the plates, and incubated for 48-72 h at room temperature[15]. Then plates were dropped with Salkowski reagent and incubated in dark condition for 30 minutes. Development of pink

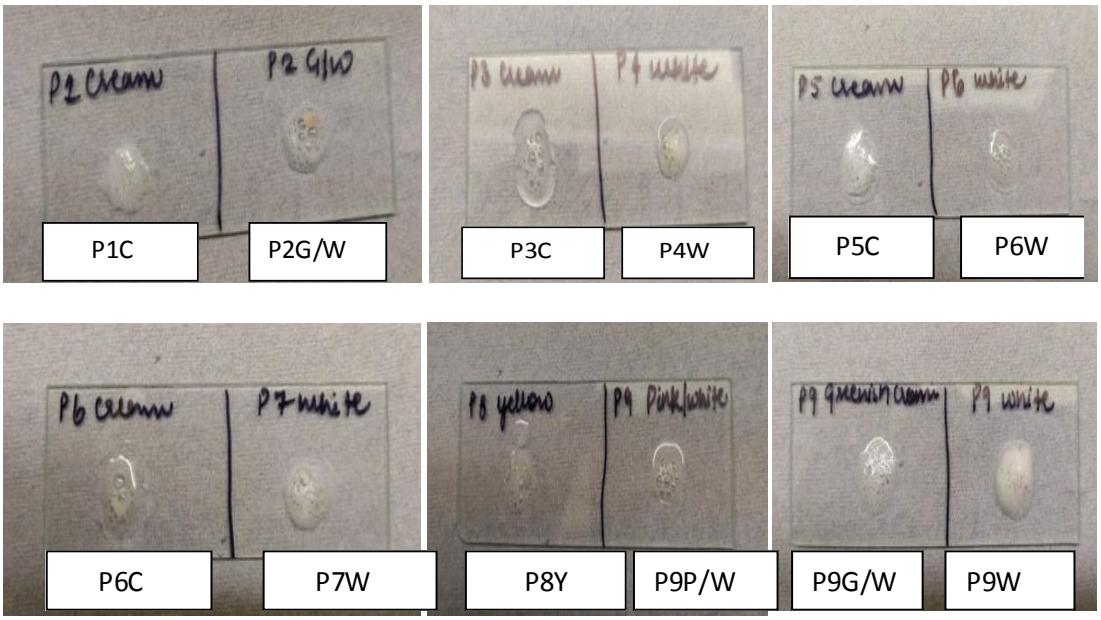
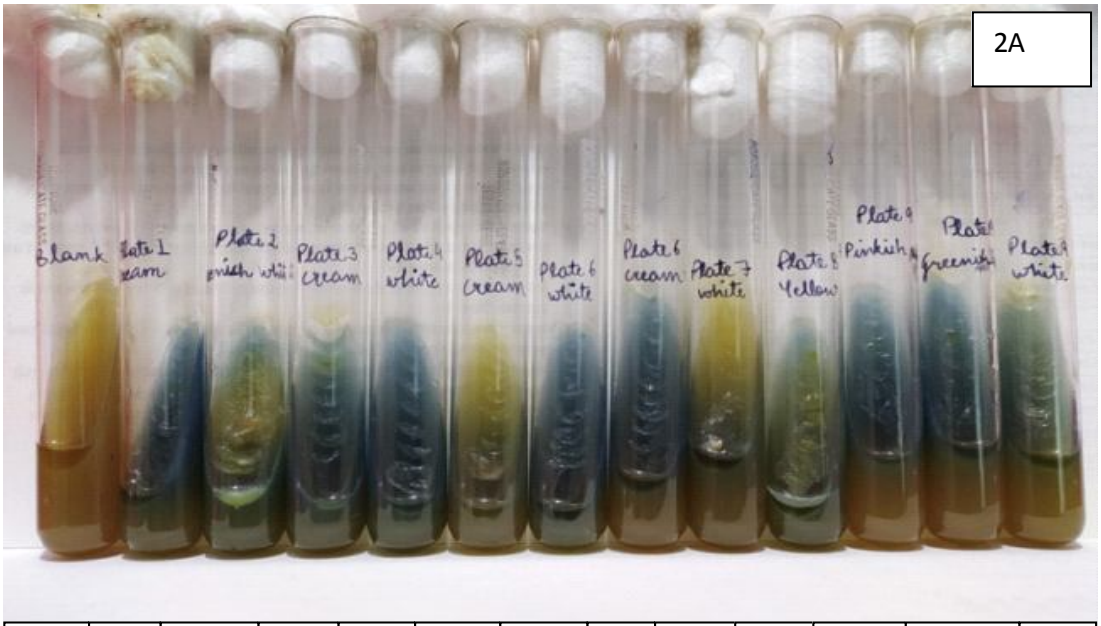


Fig. 1. Evolution of oxygen was observed by effervescences by all the colonies shows positive for catalase activity.



Control	P1C	P2G/W	P3C	P4W	P5C	P6W	P6C	P7W	P8Y	P9P/W	P9G/W	P9W

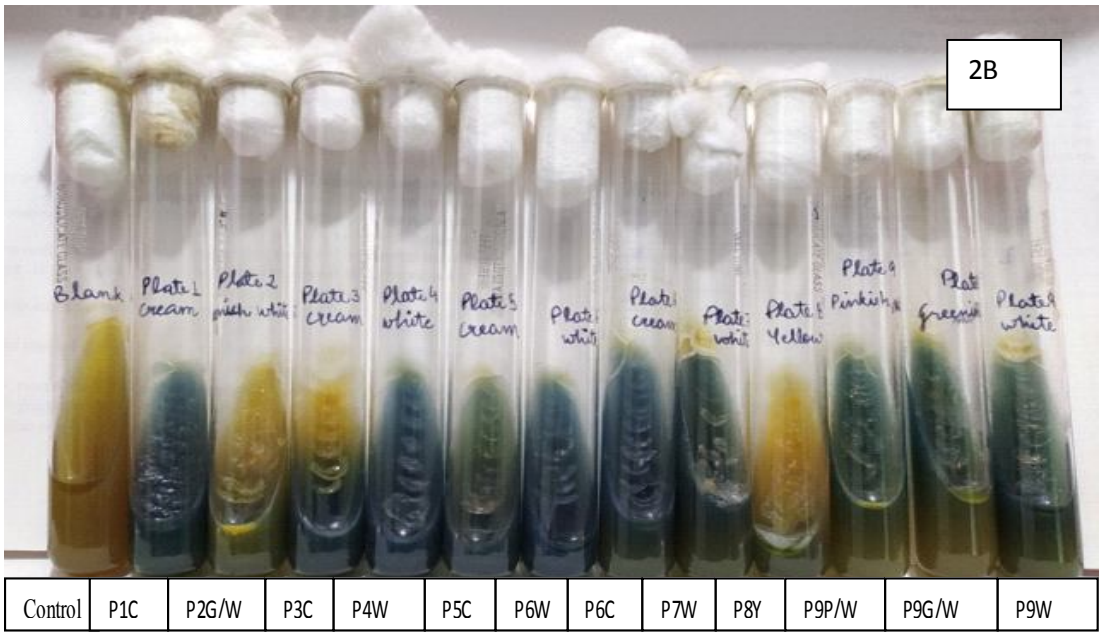


Fig. 2A and B: 2ACitrate test after 24 hours appearance of blue color shows positive citrate activity, same was observed after 48 hours in 2B.

or reddish colour around the colonies indicated IAA production.

Phosphate solubilization : The bacterial phosphate solublizing ability was assayed using National Botanical Research Institute's Phosphate growth medium (Per liter: 5g $Ca_3(PO_4)_2$, 10g glucose, 0.25g $MgSO_4.7H_2O$, 5g $MgCl_2.6H_2O$, 0.2g KCl, 0.1g $(NH_4)_2SO_4$ and 1.5% bacto-agar). Four strains per plate inoculated using sterile loop and incubated for one week at 28°C. The phosphate solubilizing isolates Formed halo zone around the bacterial colony.

Siderophore production : The traditional Chrome Azurol S agar assay was employed to conduct the siderophore production assay. Isolates were streaked on the media having HDTMA indicator of blue colour and incubated at optimal temperature of 28-30°C for 24h. Colour change from blue to orange indicated the siderophore production[3].

Plant growth promoting rhizobacteria (PGPR) are a collection of bacteria that can be established in the rhizosphere, the region of soil surrounding plant roots. These bacteria have the potential to enhance the growth of plant either direct or indirect mechanisms. A large number of bacterial species including *Azotobacter*, *Pseudomonas*, *Alcaligenes*, *Klebsiella, Arthrobacter*, *Serratia Bacillus*, *Rhizobium* and have been reported to promote plant growth. Rhizospheric soil and nodules from Indian bean were used to isolate a total of twelve colonies of distinct rhizobacteria on YEMA media through serial dilution method.

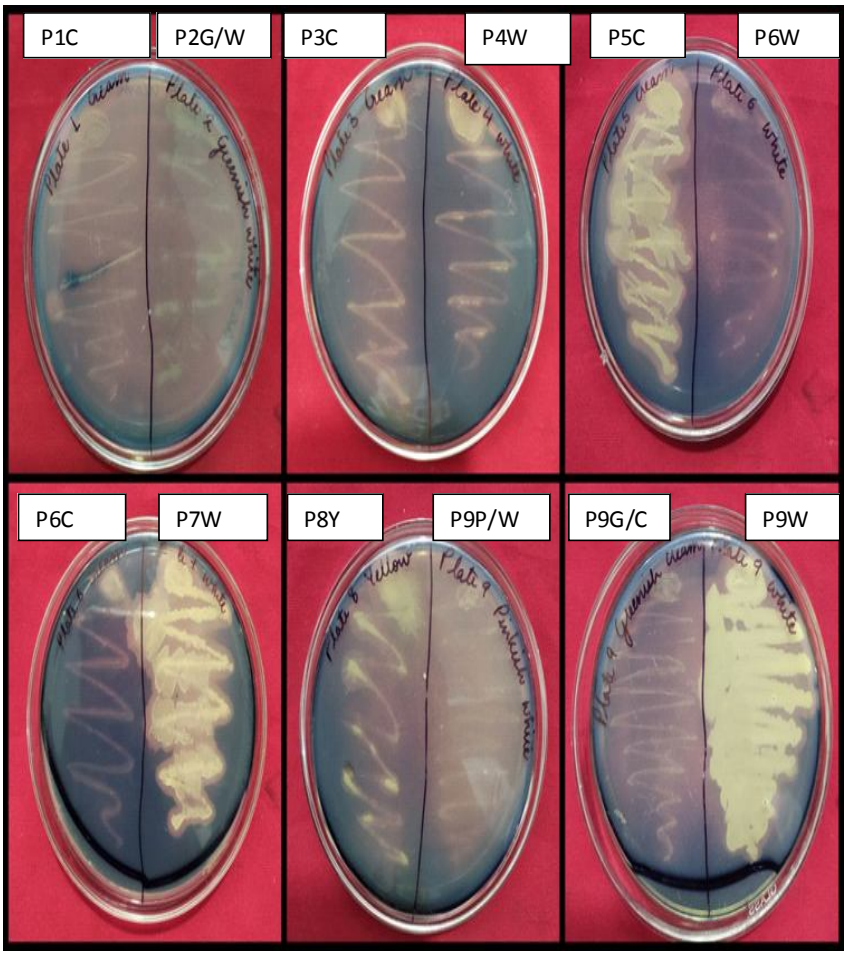


Fig. 3. All colonies show the clear zone around the line of bacterial colony for starch hydrolysis.

On particular media, each isolate was biochemically and functionally examined for its capacity to encourage plant development. The coding name for all bacterial isolates are given (table-1). The catalase test (Fig. 1), the citrate test (after 24 and 48 hours) (Fig. 2 a,b), the starch hydrolysis test (Fig. 3), and the ammonia production test (Fig. 4) were positive for all twelve bacterial isolates. IAA synthesis and siderophore formation were found in eleven isolates. Our findings of IAA production and siderophore formation in isolates are in agreement with other workers[13,16]. who observed same in *fluorescent Pseudomonas*, *Azotobacter*, *Mesorhizobium ciceri* and *Bacillus*. For phosphate solublization, six isolates were tested positive as also evidenced by the work of Mohamed *et al.,*[11], observed phosphate solublization by three bacterial strains *Bacillus subtilis*, *Serratia marcescens*

Control	P1C	P2G /W	P3C	P4W	P5C	P6C	P6W	P7W	P8Y	P9P/ W	P9G/ W	P9W

Fig. 4. Development of brown to yellow color in test tube shows the presence of ammonia production.

(PH1) and *Serratia marcescens* (PH2). Some of the above-tested isolates could exhibit more than two or three PGP traits. The subsequent identification of nine out of twelve isolates based on the analysis of the metabolic processes was made possible by additional biochemical characterization, morphological as per procedure described in Bergy‘s manual (table-1). Three isolates remained unidentified by biochemical characterization, morphological characterstics so they will be further studied by different methods in future. As this work indicated micro-organisms are good hormone producers, solublize nitrogen and phosphate, eliminate heavy metals from the soil so can be used to enhance crop production as biofertilizer.

Hernandez-Pacheco *et al.,*[7] conducted a study to assess the biofertilization potential of 315 strains isolated from different parts of *Physalis ixocarpa* plants, including stems, roots, and leaves, they identified several prevalent genera, such as *Pseudomonas*, *Microbacterium*, *Bacillus* and *Stenotrophomonas*. Notably, the endophyte strains displayed remarkable in-vitro biofertilizing activity, characterized by the synthesis of siderophores and IAA (indole-3-acetic acid), as well as the ability to solubilize phosphates. Furthermore, Cervantes-Vazquez *et al.,*[2] reported that *Pseudomonas lini* significantly enhanced stem diameter, plant height, and the fresh weight of leaves, stems and roots in tomato seedlings. Additionally, *Bacillus paralicheniformis*, *P. lini* and *Aeromonas* were found to increase the phosphate content in plants.

Synthetic nitrogen and phosphorus fertilisers were primarily used to create our current highly productive intensive farming system. Modern agriculture relies heavily on chemical fertilisers, which has resulted in a degradation of water and soil quality, making soils biologically inert and occasionally extremely salinous and contaminating both surface and ground water. In order to combat the environmental dangers posed by chemical fertilisers, it is crucial to discover affordable, environmentally friendly, and simple to use alternatives[14]. The benefits and screening of PGPR from crop plants gives a better tool to encourage their use in agriculture system. With better development and research use of PGPR can lead us towards an absolute agricultural system which is productive, sustainable, benefits environment and improves human health.

Given the information on the various positive effects of PGPR provided above, it is abundantly clear that using these PGPR is an appealing and cost-effective strategy for sustainable agriculture, instead of concentrating on inefficient methods focused on the use of chemical fertilizers. There is a need to raise awareness among farmers about the potential benefits that may be derived employing these PGPR. It is important to emphasise the commercialization of PGPR as biofertilizers. Significant advancements have been made in PGPR biofertilizer technology across the globe. Additionally, it has been demonstrated that PGPR are highly potent microbes with the potential to improve soil fertility and crop productivity, contains unique genetic components and bioactive substances having a range of applications in sustainable agriculture. In overall, we can state that the use of microbial biotechnology in agriculture has produced a number of advantages, but there are still a number of chances and difficulties that need to be investigated for the development of sustainable agriculture in the future.

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