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Isolation and Screening of Plant growth promoting rhizobacteria from agricultural soils of semi-arid Kachchh district, Gujarat, Western India

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ABSTRACT

This study aimed to identify Plant Growth Promoting Rhizobacteria (PGPR) from the rhizospheric region of crops in the Kachchh district. Bacterial isolation was performed through serial dilution techniques using different media (Himedia), and 130 bacterial cultures were isolated, with 61 morphologically distinct strains selected for further screened for PGPR traits such as Phosphate solubilization, IAA, Ammonia, Exopolysaccharide production, HCN Production, and Antifungal activity. Among these, three strains (D6, E11, and G14) exhibited the highest number of Plant Growth-Promoting Rhizobacteria traits, showed non-pathogenic characteristics in Hemolysis test, and growth rate were also estimated at a different time interval. The selected strains can further significantly contribute to the development of sustainable and eco-friendly agricultural practices by employing beneficial bacteria for plant growth, and soil fertility and it can be an alternative to chemical fertilizers for a sustainable agriculture practice.

Key words : Kachchh, PGPR, Traits, Hemolysis test, Growth rate

Introduction

Agriculture is a primary necessity for individuals all over the world. However, it heavily relies on fertilizers to increase crop yields (Kour *et al.*, 2020). Microorganisms are important for agriculture because they can make plant nutrients more accessible and lower the use of chemical fertilizers (Bhatt and Vyas 2014). Beneficial free-living soil microbes referred to as PGPR (Ordookhani, 2011), reside near the root of

plants and were extensively explored for their characteristics that enhance plant growth (Ahmad *et al.*, 2008).

The application of PGPR offers a superior choice for enhancing plant growth and managing pests and nutritional supplementation compared to conventional methods that can affect the ecosystem. Many isolates have shown a remarkable augmentation in plant stature, root extension, and the mass of both shoots and roots in plants (Saharan and Nehra

2011). PGPR has been applied to various crops, resulting in increased growth, seed development, and crop yield (Dey *et al.*, 2004).

In the present study, the isolated strains were assessed for different PGPR characteristics, including the ability to solubilize Phosphate, production of Indole-3-acetic acid (IAA), Ammonia activity, EPS (Exopolysaccharide) secretion, Antifungal activity, and HCN (Hydrogen cyanide) production. The growth rate and pathogenicity of selected strains were also determined.

Materials and Methods

Sampling area and location

Thirty-four soil samples were procured from rhizospheric region of crops using an auger from 17 locations in Kachchh, Gujarat, during the pre-monsoon and post-monsoon seasons. The rhizospheric soil was collected in sterilized containers for microbiological analyses.

Isolation and identification of isolates

Bacterial isolation was accomplished through serial dilution techniques using Nutrient agar medium, Rhizobium medium, and Kings B medium (Himedia). The bacterial isolates exhibiting various morphological appearances on agar plates were preserved on nutrient slants at 4°C.

Study on PGPR traits of isolated rhizobacterial strains

Phosphate solubilization

Estimation of phosphate was spectrophotometrically done using the chloro-stannous reduced molybdo-phosphoric acid blue method (Jackson, 1973). NBRIP (National Botanical Research Institute Phosphate) broth received one ml of each bacterial culture and was kept in incubatory shaker at 28 °C for 10 d simultaneously, along with un-inoculated media as control (Nautiyal, 1999)

Ten milliliters of each sample were collected from each conical flask at daily intervals for the duration of the 10-d incubation and centrifuged for 15 min at 10,000 rpm. The supernatant was then mixed with ammonium molybdate and stannous chloride. After a lapse of 15 min, blue color was quantified at 690 nm and the pH were also recorded. The soluble phosphate was calculated using the regression equation from the standard curve of potassium

dihydrogen phosphate (KH_2PO_4). The quantity of soluble phosphate was expressed as $\mu\text{g/l}$ over the control.

IAA production

The Salkowski method was used to measure the amount of Indole acetic acid (IAA) produced by bacterial isolates. The culture was inoculated in Luria Bertani broth supplemented with 0.1% Tryptophan and subjected to incubation at 28 °C for 48 hr. The incubation was carried out using a rotary shaker at 100 rpm. After centrifugation of the cultures at 10,000 rpm for 15 min, the supernatant was mixed with Salkowski's reagent and incubated at room temperature for 25 min. The formation of a pink coloration served as an indicator of IAA production. The amount of IAA was determined at 530 nm using a UV-VIS Spectrophotometer. IAA production concentration was assessed using the IAA standard curve (Gordon and Weber, 1951).

Ammonia production

The cultures were grown for 5 d in peptone water broth. Subsequently, 0.2 ml of the culture supernatant was combined with Nessler's reagent, and the mixture was brought to a final volume with distilled water. The transition from brown to yellow coloration indicated occurrence of ammonia and the absorbance was measured at 450 nm. Subsequently, the ammonia concentration was ascertained by employing the ammonium sulfate standard curve, which encompassed the range of 0.1-1 $\mu\text{mol/ml}$ (Cappuccino and Sherman, 1992).

EPS (Exopolysaccharide) production

The isolated strains were inoculated in LB broth supplemented with 5% sucrose and incubated at 35 ± 2 °C for 5 d at 200 rpm. After centrifugation, EPA was extracted by adding pre-chilled acetone with supernatant. The extracted was filtered using a 0.45 μm nitrocellulose filter through Millipore filtration. The precipitated EPS was dried overnight at 80°C and weighed (Mody *et al.*, 1989).

Antifungal activity

The dual culture assay was used to assess the antifungal potential of bacterial isolates against *Candida albicans*. The fungal and bacteria were streaked in opposite sides of the plate and incubated at 28 ± 2 °C for 96 hr. The inhibitions of fungal mycelium found around the bacterial colony were observed followed

by the determination of subsequent measurement of size of the inhibition zone (Gopalakrishnan *et al.*, 2011).

Hydrogen cyanide (HCN) Production

The sulfocyanate colorimetric method was used for qualitative estimation. Kings' B agar supplemented with 4.4 g/l glycine was used to culture bacterial isolates. Filter paper soaked in a 1% picric acid solution was placed in petri plate sealed by Para-film followed by the incubation for 48 hr at 28 ± 2 °C. The presence of HCN activity was determined by the change in color from yellow to light brown or brick red (Lorck, 1948; Abd *et al.*, 2019).

Hemolysis test

The Blood Agar Hemolysis test involves using modified Blood Agar media with 5% defibrinated sheep blood. After sterilization, the media is mixed with sterile blood, avoiding bubbles, and poured into sterile plates. Once solidified, test bacteria are aseptically inoculated onto the plates, followed by incubation at 37 °C for 18-24 hr. Following incubation, the plates are assessed for hemolysis patterns (α , β , or γ) (Buxton, 2005).

Growth rate of culture

The growth rates of cultures were evaluated using different forms, including single, duplicate, and consortium setups. For this experiment, the cultures were grown in LB broth and positioned in a shaker and further, the optical density was measured at 600 nm using a UV spectrophotometer at different time intervals (18, 24, 48, 72, and 96 hr) (Matlock, 2017).

Statistical analysis

All the data descriptive statistical analyses were performed utilizing Microsoft Excel software.

Results and Discussion

In this study, a comprehensive investigation was conducted to explore PGPR from soil samples. Initially, 130 bacterial strains were obtained from the soil samples. Among these, 61 strains were carefully selected based on their distinct morphological characteristics for further analysis. These 61 strains underwent rigorous screening to assess their PGP traits. After a thorough examination, three bacterial strains, namely D6, E11, and G14, stood out as they displayed the highest number of PGP traits. As a

result, these three selected strains were chosen for subsequent experiments, aiming to delve deeper into their potential to promote plant growth and further explore their agricultural applications. The findings from this research could significantly contribute to the development of sustainable and eco-friendly agricultural practices by employing beneficial bacteria to enhance both plant growth and the yield of crops.

PGPR traits of isolated strains

PGPR is vital in protecting and augmenting plant growth through a diverse array of mechanisms. These encompass the production of phytohormones such as IAA, which encourages root growth and overall plant development. Additionally, PGPR assists in nutrient management by solubilizing phosphates, antioxidant production, and making essential nutrients more accessible to plants thereby improving nutrient uptake and utilization (Ahmad *et al.*, 2008). The beneficial impact of PGPR and their interactions with plants hold immense potential for application in ecological and sustainable agriculture practices (Wang *et al.*, 2020). By reducing the reliance on chemical inputs, PGPR offers an eco-friendly and economically viable approach to enhance crop productivity while promoting environmental health. The study aimed to isolate and screen bacterial strains based on various PGPR traits. Through rigorous screening, three bacterial strains that exhibit promising plant growth-promoting characteristics were identified by paving the way for further investigation and potential utilization in practical agricultural applications.

In soil, free-living P-solubilizing bacteria play a crucial role in releasing phosphate ions derived from slightly soluble inorganic and organic phosphate compounds. This process contributes to the increased availability of phosphate in soil, making it more accessible to plants (Artursson *et al.*, 2006). The isolates tested in the study demonstrated varying degrees of phosphate solubilization, with G14 > D6 > E11 order, where G14 showed the highest phosphate release with a value of 4897.73 ± 25.53 mg/l, and E11 exhibited the lowest value at 4131 ± 20 mg/l. During the experiment, a noticeable pH reduction was observed in the culture broths. This pH decline signifies an elevated level of solubilization resulting in more phosphorus contents being released into the broth. The ability of *A. rhizosphaerae* strain BIHB 723 to solubilize phosphate varied depending on the

specific phosphate substrates, which aligns with findings reported for other bacterial species such as *Azospirillum brasiliense*, *Citrobacter* spp., *Pantoea agglomerans*, *Pseudomonas corrugata*, and *Rhizobium* spp (Patel *et al.*, 2008). These findings emphasize P-solubilizing bacteria's important role in boosting soil fertility and enhancing nutrient availability for plants.

Bacteria that produce IAA have a positive impact on root elongation and overall plant growth (Patten and Glick, 2002). In the present study, IAA production was measured in different bacterial strains, and it was found that strain D6 exhibited the highest IAA production at 76.97 ± 1.63 mg/l, while the E11 strain showed the lowest IAA production at 64.59 ± 1.53 mg/l. Comparing the results (Gopalakrishnan *et al.*, 2011), it was observed that isolates SRI-211 and SRI-229 demonstrated even higher IAA production, with values of 8.06 and 8.86 μ g/ml, respectively, indicating their potent ability to produce this phytohormone. Except SRI-305, the majority of other isolates generated IAA within the 2 to 4 μ g/ml range. Hence, the disparity in IAA production among bacterial strains highlights the significance of carefully selecting and employing strains with greater IAA production to efficiently boost plant growth. Certain bacteria producing significant levels of IAA are valuable candidates for potential application in agricultural practices aimed at promoting root growth and improving overall plant health.

Ammonia-producing PGPR inoculation demonstrates favorable impacts on *Zea mays* (corn) growth, promoting higher root length, shoot length, and biomass (Marques *et al.*, 2010). In this study, all tested bacterial strains exhibited positive outcomes in ammonia production. Out of these varieties, the D6 strain exhibited the greatest level of ammonia production, with a concentration of 38.2 mg/l, while the G14 strain displayed the lowest production at 35.36 ± 0.16 mg/l. This further supports the positive impact of ammonia-producing PGPR on plant growth and development. In a similar study, 28 different isolates showed ammonia production. Among these isolates, the KB12 strain showed the highest ammonia production, with a concentration of 4.72 μ mol/ml (Goswami *et al.*, 2014). This finding is consistent with the importance of ammonia production by PGPR in promoting plant growth and highlights the potential variation in ammonia production among different bacterial strains. These re-

sults underscore the significance of ammonia-producing PGPR as valuable agents for promoting plant growth and enhancing agricultural productivity.

The extreme production of exopolysaccharides (EPS) by plant growth-promoting rhizobacteria (PGPR) signifies their inherent protective mechanism against metal toxicity while thriving in stressful environments (Karthik *et al.*, 2016). In the current study, significant EPS production was observed in all the bacterial isolates. The maximum EPS activity was recorded in the E11 strain, with a concentration of 7479.67 ± 0.88 mg/l, while the G14 strain displayed lower activity with a concentration of 90.33 ± 0.66 mg/l. The EPS acts as a protective shield, enabling the bacteria to survive and support plant growth even in challenging environments. Understanding the EPS production capability of PGPR isolates can aid in the selection of strains with enhanced stress-tolerance properties for applications in bioremediation and sustainable agriculture.

Antifungal potential was assessed using the dual culture assay, and the top seven promising isolates were tested against the pathogen *Macrophomina phaseolina*. Notably, SRI156 and SRI158 among these isolates exhibited remarkable pathogen inhibition. The observed inhibition might be due to the SRI isolates releasing hydrolytic enzymes or antibiotics that diffused through the growth medium (Gopalakrishnan *et al.*, 2011). Similarly, three selected isolates in the present study demonstrated antifungal activity against *Candida albicans*. Among these isolates, D6 exhibited an inhibition zone of 3.03 ± 0.09 mm, E11 showed 4.83 ± 0.12 mm, and G14 displayed the highest inhibition with 8.97 ± 0.09 mm respectively. The production of antifungal compounds by these PGPR isolates can be beneficial in suppressing fungal diseases and protecting plants from pathogenic attacks. Further investigations into the mechanisms of antifungal action employed by these isolates could provide valuable insights for developing biocontrol agents to manage fungal diseases in agriculture and other relevant fields. In the current study, the three bacterial strains tested did not exhibit any color change in filter papers, indicating the absence of HCN production. Therefore, the HCN production test yielded negative (-) results for all three strains. A previous study focusing on *Bacillus xiamenensis* PM14 also reported no activity for HCN production (Amna *et al.*, 2020). The comprehensive results, including the absence of HCN pro-

duction, are provided in Table 1 and Figure 1.

The hemolysis test outcomes from this study indicated the presence of α hemolysis on the plates, signifying the non-pathogenic nature of all three strains. (Figure 2). This non-pathogenic nature is further supported by the absence of any hemolytic activity observed for the isolates KB-10 and KB-25 in previous studies (Al-Garni *et al.*, 2019). The absence of hemolytic activity in previous research suggests that these bacterial strains do not cause damage to red blood cells and are considered non-pathogenic. The growth rate of the isolated bacterial strains was investigated in different experimental sets, including single strains (D6, E11, G14), double combinations (D6+E11, E11+G14, D6+G14), and a consor-



Fig. 2. Haemolysis pattern of the PGPR isolates

tium of all three strains (D6+E11+G14). All of these sets exhibited characteristic growth rates during the observation period. However, after 72 hr, the bacterial numbers in all the sets started to decrease due to the decline phase (Figure 3).

Table 1. Results of PGPR traits of isolates

Cultures	PGPR Traits					
	Phosphate solubilization (mg/l)	IAA production (mg/l)	Ammonia activity (mg/l)	EPS (mg/l)	Antifungal (mm)	HCN
D6	4356±39.86	76.97±1.63	38.59±0.19	1670.33±0.88	3.03±0.09	Negative
E11	4131±20	64.59±1.53	36.33±0.49	7479.67±0.88	4.83±0.12	Negative
G14	4897.73±25.53	69.46±1.14	35.36±0.16	90.33±0.66	8.97±0.09	Negative

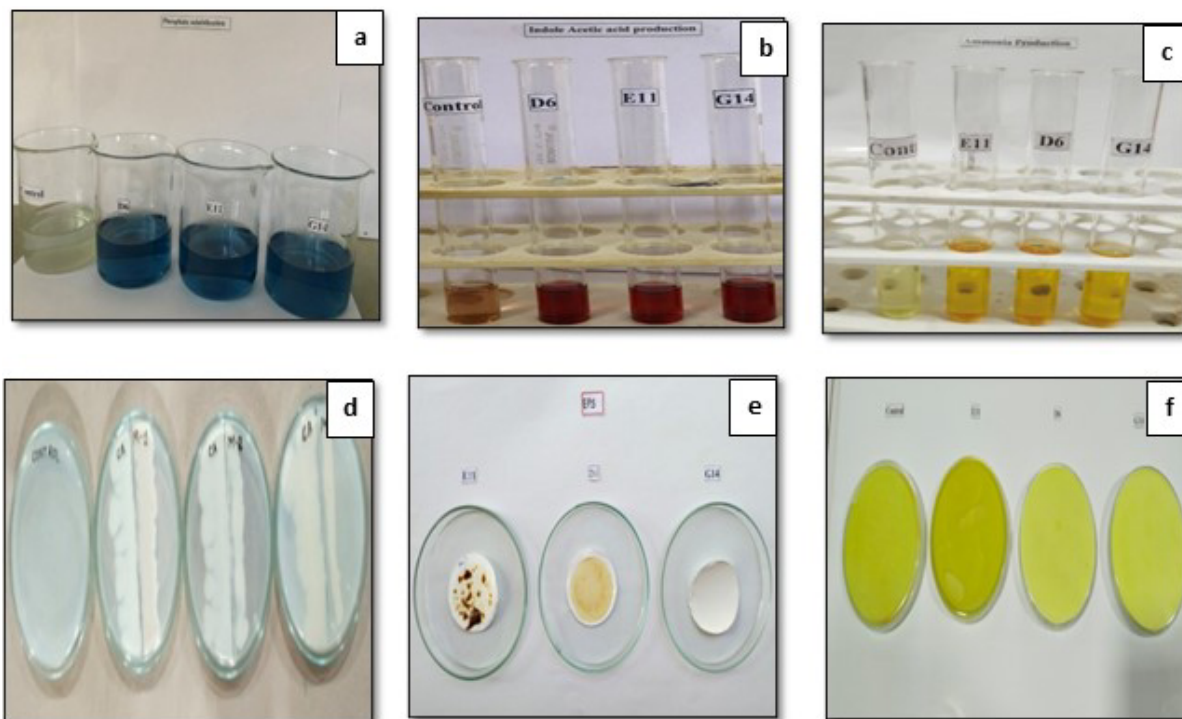


Fig. 1. Isolates showing PGP activities of Phosphate solubilization (a); Indole Acetic Acid production (b); Ammonia production; (c); Antifungal activity (d); EPS production (e) and HCN production (f)

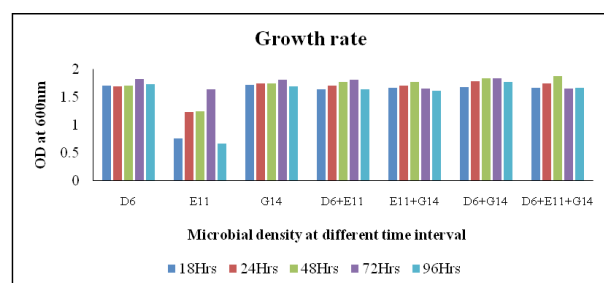


Fig. 3. Bacterial growth rate at different time interval (in hrs)

Conclusion

Excessive use of chemical fertilizer led to complete disturbance in the soil ecosystem and a challenge to attain sustainable crop production. Therefore, it is compulsory to switch from inorganic to organic agricultural practices for the well-being of future agricultural productivity. Hence in this modern era, the good nature of PGPR can be introduced in agricultural practices and a better alternative to chemical fertilizers. PGPR display its unique characteristic such as hormonal secretion, and uptake of soil macro, secondary, and micronutrients. The present study focuses on Plant growth promoting rhizobacteria (PGPR) isolation and screened for PGPR characteristics. The best three strains (D6, E11, and G14) were selected based on their PGPR traits, pathogenicity test, and growth rate. Further research significantly contributes to the development of sustainable and eco-friendly agricultural practices by employing beneficial bacteria to improve plant productivity and soil fertility and it can be an alternative to chemical inputs for a sustainable agriculture practice.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Authors' Contribution

The author KK has conceptualized and designed the experiments, author MRS and IMP has conducted

the experiments and wrote the original draft. Authors GJ and KDS has reviewed and edited the manuscript. All the authors read and approved the final manuscript for publication. All the authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Data Availability

All datasets generated and/or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not Applicable.

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