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Screening and optimization of indole-3-acetic acid production by *Rhizobium* sp.

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Abstract: PGPR plays a crucial role in significantly enhancing plant growth. PGPR biofertilizers are indispensable for agricultural practices, providing plants with essential phytohormones, soluble phosphate, bioavailable iron, and nitrogen-fixing capabilities. Bacteria, specifically *Rhizobium* sp., were isolated from the rhizospheric soil of rice crop fields. Rigorous evaluation of the bacterial strains revealed that *Rhizobium* sp. demonstrated the highest IAA production (119.18 ±2.42 µg mL-1) under specific conditions, such as 0.2% L-tryptophan, 2.0% NaCl, at a pH of 8.0, after a 10-day incubation period at 35°C. The findings from this study provide valuable insights into optimizing conditions to maximize IAA production by PGPR. These optimized parameters can be readily applied in agricultural practices to enhance crop yields significantly, especially in stress-prone environments.

IndexTerms **- PGPR, IAA, *Rhizobium* sp., L-tryptophan, optimization, sustainable agricultural practices, stress environments**

I. INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are naturally spread throughout the soil system and improve plant growth by phosphate solubilization, phytohormones synthesis, nitrogen fixation, and siderophore production (Glick, 2012; Ahemad & Kibret, 2014; Gupta *et al*., 2018). Moreover, certain PGPR strains indirectly protect plants by encouraging defence mechanisms against pathogenic aggression (Pieterse *et al*., 2003). Primarily, plant species are symbiotically associated with PGPR including *Acinetobacter*, *Agrobacterium*, *Aeromonas*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Klebsiella*, *Micrococcus*, *Pseudomonas*, *Rhizobium*, *Thiobacillus*, and *Xanthomonas* (Habibi *et al*., 2014). These bacterial genera often lay out in the soil and make symbiotic associations with specific host plants or reside as free-living. Their agricultural potential has gradually enlarged since the search for eco-friendly alternatives to chemical fertilizers and biocides (Nathiya *et al*., 2020). Therefore, scientific communities endorse PGPR as sustainable biofertilizers to improve nutrient availability (N, P, K, and micronutrients), significant plant growth, and enhanced yield (Zahid *et al*., 2015). Undeniably, rhizobia were considered a commercially viable strain worldwide to enhance plant growth (Marks *et al*., 2015). These bacteria also produce plant growth-promoting substances, e.g., hormones such as Indole-3-acetic acid (IAA), known as physiologically active phytohormones that promote effective plant growth (Chandra *et al*., 2018). IAA is secreted by rhizobacteria that enlarge the root system, e.g., root size, root hairs, and magnitude of lateral root (Fierro-Coronado *et al*., 2014; Jaiswal *et al*., 2018). This action improves the development and yield of plants by maximum utilization of soil resources and acquisition of nutrients via the root system (Ramos *et al*., 2018).

The production of IAA by PGPR is influenced by various factors such as type of species, culture conditions, growth phase, and substrate availability (Mohite, 2013). Plant roots release organic compounds, viz., L-Tryptophan, by which rhizobacteria synthesize IAA under natural conditions. Nevertheless, to meet the commercial level of IAA production, the strain must be supplemented with L-tryptophan. The conversion of L-Tryptophan to IAA in rhizobacteria involves deamination, decarboxylation, and hydrolysis reactions. The indole-3-pyruvic acid pathway is the primary path for IAA synthesis (Lee *et al*., 2004). The pathway differs among different species or genera. IAA production by rhizobacteria often helps plants withstand biotic and abiotic stress conditions. However, limited scientific evidence shows a relationship between stress and auxins (Ahmad *et al*., 2005). However, environmental factors such as pH, temperature, and tryptophan contribute to increased crop yields (Lebrazi *et al*., 2020; Spaepen *et al*., 2009). Hence, the present research was conducted to isolate and identify PGPR strains with IAA-producing ability and its optimization.

II. NEED OF THE STUDY

Rhizobia are a commercially viable strain that enhances plant growth by producing plant growth-promoting substances, such as hormones, such as IAA. It enlarges the root system, which improves plant development and yield by maximizing soil

resource utilization and nutrient acquisition. Hence, the optimization of indigenous *Rhizobium* sp. derived-IAA could contribute to enhancing crop yield and encouraging the plant defence system against pathogens in crop fields.

III. RESEARCH METHODOLOGY

The present research work was focused on the isolation of PGPR *Rhizobium* sp. and its optimization for significant IAA production at various parameters. A research methodology is systematically presented below:

3.1 Sample collection and isolation of *Rhizobium* sp.

The Rhizopheric soil of the rice crop fields from the agriculture field of Birkona Village under Belha Block of Bilaspur District (22.14679, 82.16287) was aseptically collected in sterile polythene pouches and brought to the laboratory to isolate *Rhizobium* sp. The *Rhizobium* Medium, consisting of (gL-1) Mannitol-10.0, dipotassium phosphate-0.5, magnesium sulphate-0.20, yeast extract-1.0, sodium chloride-0.10, and agar-20.0, was used for isolation of *Rhizobium* sp. at a final pH of 6.8 ±0.2 (Subba Rao, 1977). The colony morphology, gram stain, motility test, citrate utilization, indole production, methyl red, nitrate reduction, oxidase, Voges Proskauer, carbohydrate utilization, starch hydrolysis and gelatin hydrolysis test were performed, as mentioned by Aneja (2003).

3.2. Screening of *Rhizobium* sp. for IAA production

The *Rhizobium* sp. was screened for IAA production using the Salkowski assay as protocol defined by Bric *et al*. (1991). The pure culture of *Rhizobium* sp. was grown in Rhizobium broth media at 28 ± 2 °C for seven days at 150 rpm. Later, the bacterial culture was centrifuged (at 10000 rpm) for 10 min at 4°C. This supernatant was considered a crude IAA. One mL of crude IAA was mixed with 2.0 mL of Salkowski reagent (consisting of 1.0 ml, 0.5 mol L^{-1} Iron chloride in 49.0 ml of 35% perchloric acid). The reaction mixture was then incubated for 30 min. Further, the absorbance was measured at 530 nm. The concentration of IAA was calculated using an equation generated by a standard IAA curve.

3.3. Partial purification of crude IAA

The crude IAA was partially purified using the protocol mentioned by Lebrazi *et al*. (2020). The crude IAA was acidified to pH 2.0 using HCl (1.0 N). Afterwards, acidified crude IAA was extracted twice by ethyl acetate. The extracted IAA fraction was then evaporated using a rotatory evaporator at 40 °C. The concentrated IAA extract was mixed with 1.0 ml of methanol and kept at 20 °C in the dark. Further, the partially purified IAA was subjected to thin-layer chromatography (TLC) with Silica gel Gf 254 (0.25 mm thick layer). A mixture of n-butanol: ethyl acetate: ethanol: water at a ratio of 3:5:1:1 was used as an organic solvent for TLC. IAA was identified under UV light (at 254 nm) by spraying Ehmann reagent (Ehmann, 1977).

3.4. Optimization of IAA production by *Rhizobium* sp.

The Effect of L-tryptophane (0.1, 0.2 and 0.3 %), pH (6,7 and 8), temperature (30, 35 and 40 °C) incubation period (5, 10 and 15 days), and salinity in terms of NaCl (1, 2 and 3 %) on IAA production by *Rhizobium* sp. were examined.

All data was entered and processed using Microsoft Excel 2021. The experiments were conducted in triplicate, and the mean value and standard deviation were determined using the relevant built-in functions in Microsoft Office Excel.

IV. RESULTS AND DISCUSSION

The present research was conducted to isolate and identify PGPR Rhizobium sp. and optimize its IAA production. Three IAA-producing bacteria were isolated. The Colony, Microscopic and Biochemical characteristics of bacterial isolates confirm the *Rhizobium* Genera (Table 1).

Table 4.1 Colony, Microscopic and Biochemical characteristics of *Rhizobium* sp.

S. No.	Characteristics	Observation
Colony Characteristics		
1.	Form	Round
2.	Elevation	convex
3.	Texture	smooth
4.	Color	white
Microscopic Characteristics		
5.	Gram Stain	-
6.	Shape	Rod
Biochemical Characteristics		
7.	Motility	+
8.	Indole Production	-
9.	Citrate Utilization	+
10.	Nitrate Reduction	+
11.	Methyl Red	-
12.	Voges Proskauer	+
13.	Oxidase	+

14.	Starch Hydrolysis	-
15.	Gelatin Hydrolysis	-

4.1 Optimization of IAA production by *Rhizobium* sp.

Rhizobium sp. exhibited significant IAA production, which was further evaluated to optimize it. The results were cumulatively plotted as a bar diagram in Fig. 1.

4.1.1. L-Tryptophane

Rhizobium sp. was supplemented with different concentrations of L-tryptophan (01, 0.2, and 0.3 %), and the maximum IAA production (µg mL^{-1} ±SD) of 112.57 ±2.64 was observed with 0.2 %, followed by 64.98 ±1.95 with 0.3 % and 42.86 ±1.59 with 0.1%. Lebrazi *et al*. (2020) revealed that 0.2% of L-Tryptophan exhibited a maximum of 116.42 µg L^{-1} of IAA production. Likewise, Ghosh *et al*. (2013) have also reported 0.2% of L-Tryptophan for a significant level of IAA production by *Rhizobium* sp.

4.1.2. NaCl

The *Rhizobium* sp. was grown with different concentrations of NaCl (1, 2 and 3 %) and the maximum IAA production (µg mL^{-1} ±SD) of 78.56 ±2.06 was recorded with 2.0 %, followed by 63.71 ±1.77 at 1 % and 61.78 ±1.82 at 1.0 %. Lebrazi *et al*. (2020) mentioned that a higher IAA was observed produced at 2.0 % of NaCl. Further, Wang *et al*. (2001) noted that gradual increase in NaCl concentration mitigates the IAA production from above 2.0 %.

4.1.3. pH

The *Rhizobium* sp. exhibited an indole-3-acetic acid (IAA) production of 108.53 ± 3.05 µg mL^{-1} at pH 8.0, followed by 96.38 ±2.18 µg mL^{-1} at pH 7.0 and 44.12 ±1.34 µg mL^{-1} at pH 6.0. According to the literature, the maximum IAA production by *Rhizobium* sp. has been noted at pH 9.0 (Chandra *et al*., 2018; Raut *et al*., 2017). Additionally, Thokal *et al*. (2013) found that the synthesis rate of IAA increased from neutral to slightly alkaline pH.

4.1.4. Incubation period

Rhizobium sp. was fermented for 5, 10, and 15 days. The maximum IAA production (µg mL-1 ±SD) of 119.18 ±2.42 was noted after ten days of incubation, followed by 73.26 ±2.07 and 42.67 ±1.31 after 15 and 5 days of incubation, correspondingly. In comparison, Lebrazi *et al*. (2020) noticed the maximum IAA production after nine days of incubation with *Rhizobium* sp.

4.1.5. Temperature

Rhizobium sp. underwent fermentation at varying temperature intervals (30°C, 35°C, and 40°C). The highest indole-3-acetic acid (IAA) production of 102.54 ±3.17 µg mL^{-1} was observed at 35°C, followed by 69.73 ±2.28 at 40°C and 52.14 ±1.69 at 30°C. Research by Sudha *et al*. (2012) indicates that the optimal temperature for IAA production with *Rhizobium* sp. is 37°C. Khanna et al. (2010) also noted that the maximum IAA production by plant growth-promoting bacterium *Streptomyces* sp. occurred at 30°C.

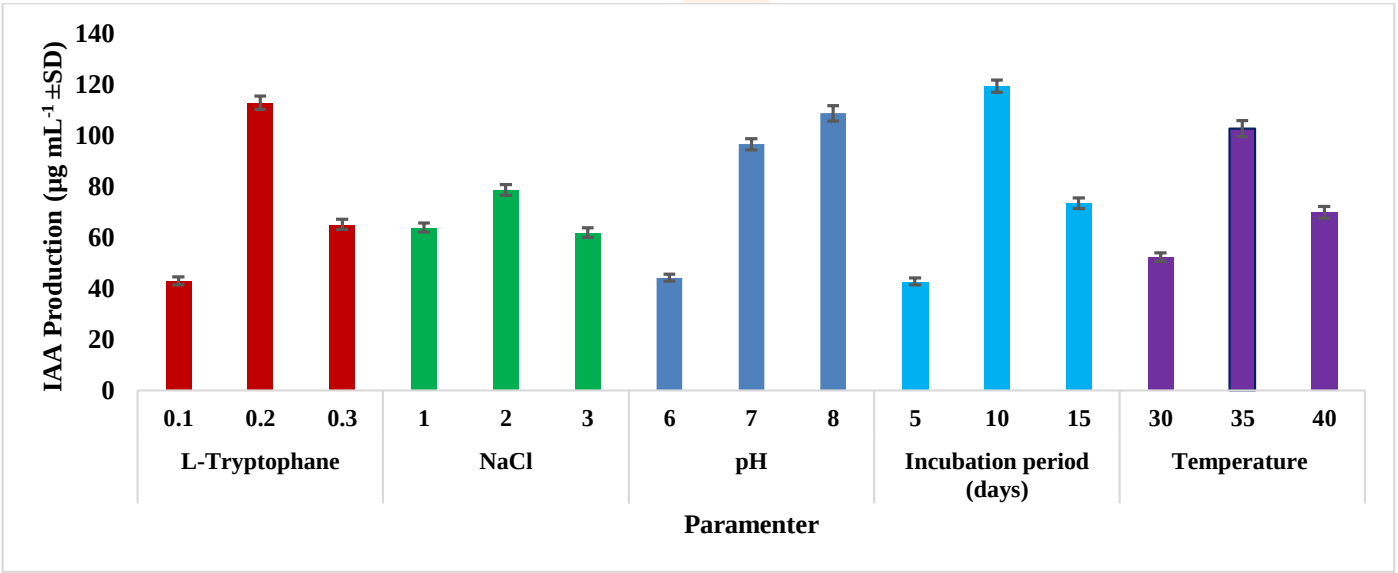

Fig 1. Optimization of IAA production by *Rhizobium* sp. at various parameters

We achieved a maximum of 119.18 ±2.42 µg mL-1 of IAA production using a medium supplemented with 0.2% L-tryptophane and 2% NaCl. This was done over ten days at 35°C and a pH of 8.0. In addition to IAA, *Rhizobium* sp. also synthesizes siderophores that promote root development and increase nutrient uptake. The literature claims that they improve plant resilience against pathogens. Consequently, the use of *Rhizobium* as PGPR offers an eco-friendly approach to improving crop productivity and environmentally conscious agriculture by reducing reliance on chemical fertilizers.

V CONCLUSION

Our study on the production of IAA by PGPR and the optimization of various parameters such as pH, temperature, incubation time, salinity, and L-tryptophan concentration has revealed the significant impact of these factors on IAA synthesis. It is crucial to optimize these parameters to maximize IAA production, which is essential for promoting plant growth and development in agriculture. Our findings demonstrate that PGPR strains can produce substantial amounts of IAA under specific pH levels, temperatures, incubation periods, salinity, and L-tryptophan concentrations. The optimal conditions identified in this study provide a framework for enhancing the efficiency of IAA production, with potential applications in agricultural practices to support sustainable crop growth. This research contributes to our understanding of microbial hormone production and offers practical opportunities for developing biofertilizers and sustainable agriculture practices.

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