



Isolation, Screening, Characterization of *Azospirillum* spp from Rhizospheric Soil and Checking its Plant Growth Promoting Ability

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Abstract – The present study was conducted to isolate, screen, and characterize plant growth promoting *Azospirillum* spp. from rhizospheric soil and evaluate their plant growth promoting (PGP) traits and their effect on seed germination and early plant growth under laboratory conditions. Rhizospheric soil samples were collected and *Azospirillum* spp. were isolated using selective media. The isolates were screened and characterized based on morphological and biochemical characteristics. The selected isolates were evaluated for plant growth promoting traits including Indole-3-acetic acid (IAA) production, phosphate solubilization, ammonia production, hydrogen cyanide (HCN) production, and cellulase activity using standard qualitative and quantitative methods. The effect of the isolates on seed germination and early plant growth was also evaluated under laboratory conditions. Three isolates from soil sample (S1, S2 and S3) were obtained and all isolates showed multiple plant growth promoting traits. IAA production ranged from 33.71-42.37 ppm, with isolate S1 showing the highest production. Phosphate solubilization ranged from 2.11-3.66 µg/ml, with S3 showing the highest activity and significant difference among isolates. Ammonia production and HCN production were positive in all isolates, and cellulase activity was also observed, indicating their multifunctional plant growth promoting potential. Although the quantitative values were moderate, the isolates showed consistent plant growth promoting traits. Seed germination and early plant growth studies indicated improved growth parameters in treated seeds compared to control. In conclusion, the isolated *Azospirillum* spp. demonstrated multiple plant growth promoting traits and showed potential for use as biofertilizers in sustainable agriculture due to their combined plant growth promoting activities.

Keywords – Soil Microbial Communities, Plant Growth Promoting Rhizobacteria (PGPR), *Azospirillum* spp., Rhizosphere, Indole-3-Acetic Acid (IAA), Phosphate Solubilization, Ammonia Production, Hydrogen Cyanide (HCN), Cellulase Activity, Wheat Seed Germination, Bioinoculant, Sustainable Agriculture.

I. INTRODUCTION

Soil is a dynamic and biologically active system that supports plant growth through complex interactions among physical, chemical, and biological components. The rhizosphere, influenced by plant root exudates, hosts a diverse microbial community that plays a key role in nutrient cycling and plant health (Rani et al., 2023; Timofeeva et al., 2023). Among these microorganisms, Plant Growth Promoting Rhizobacteria (PGPR) enhance plant growth by improving nutrient availability, producing phytohormones, and protecting plants from environmental stresses (Grover et al., 2022; Sun et al., 2025). The genus *Azospirillum* is an important group of PGPR associated with cereal crops like wheat. These bacteria promote plant growth through nitrogen fixation and the production of growth regulators such as indole-3-acetic acid (IAA), along with other beneficial metabolites (Fukami et al., 2018; Cruz-Hernández et al., 2022). They also contribute to phosphate solubilization, siderophore production, and improved root architecture, leading to enhanced nutrient uptake (Timofeeva et al., 2023; Sarkar et al., 2023). In addition, *Azospirillum* spp. help plants tolerate abiotic stresses such as salinity and drought, further improving crop productivity (Naqqash et al., 2022; Khumairah et al., 2022) Due to these



multifunctional traits, *Azospirillum* spp. are considered promising biofertilizers for sustainable agriculture and reduced chemical input (Aasfar et al., 2021; Yasuda et al., 2022). Therefore, evaluating their plant growth-promoting potential, particularly during seed germination and early seedling stages, is essential for improving wheat cultivation.

II. MATERIAL AND METHODS

2.1. Sample Collection

Rhizospheric soil samples were collected from agricultural fields in Guhagar (Ratnagiri, Maharashtra) and Gurdaspur (Punjab). Three samples were designated as S1 (Guhagar), S2 (Guhagar), and S3 (Punjab). Samples were transported to the laboratory for further analysis. (Kavya et al., 2023) (Rajesh et al., 2023).

2.2. Enrichment and Isolation of *Azospirillum* Spp.

One gram of each soil sample was inoculated into 15 mL Nitrogen-Free Bromothymol Blue (NFB) semi-solid medium and semi-solid *Azospirillum* medium (pH 6.5) and incubated at 25°C for 5-7 days. Formation of a characteristic subsurface pellicle indicated the presence of diazotrophic bacteria. Enriched cultures were streaked onto selective *Azospirillum* agar plates and incubated at 37°C for 5-7 days. Distinct colonies were purified by repeated streaking. (Ayyaz, K., et al. (2016)) (Avinash, M., & Swamy, M. (2018) (Rajesh, C., et al. (2023).

2.3. Morphological and Biochemical Characterization

Isolates were characterized based on colony morphology and Gram staining. Biochemical tests included oxidase, catalase, growth in 3% NaCl, Voges Proskauer (VP) test (with 2% glucose), and carbohydrate fermentation (glucose, mannitol, galactose) using Andrade's indicator. (Collee et al., 1996) (Jain et al., 2021).

2.4. Determination of Plant Growth Promoting Traits

2.4.1. Indole-3-acetic acid (IAA) production:

Isolates were grown in Luria broth supplemented with tryptophan (1 mg/mL) at 37°C (120 rpm) for 48 h. After centrifugation (3000 rpm, 10 min), 1 mL supernatant was mixed with 2 mL Salkowski's reagent and incubated for 30 min. Absorbance was measured at 530 nm, and IAA concentration was determined using a standard curve. (Fukami et al., 2018) (Timofeeva et al., 2023).

2.4.2. Phosphate Solubilization

Qualitative assessment was performed on Pikovskaya's agar by observing halo zone formation after 7 days at 30°C. Quantitative estimation was conducted using buffered disodium phenyl phosphate substrate, followed by color development with 4-aminoantipyrine and potassium ferricyanide. Absorbance was recorded at 530 nm, and enzyme activity was calculated from a phenol standard curve. (Mukherjee & Dutta, 2019) (Timofeeva et al., 2023).

2.4.3. Ammonia production:

Isolates were inoculated in 4% peptone broth and incubated for 24 h. Nessler's reagent was added; yellow to brown coloration indicated ammonia production. (Cappuccino & Sherman) (Jain et al., 2021).



2.4.4. HCN Production:

Isolates were streaked on nutrient agar supplemented with glycine (4.4 g/L). Picric acid-impregnated filter paper was placed in the lid and incubated at $28\pm 2^{\circ}\text{C}$ for 2-4 days. Color change from yellow to brown indicated HCN production. (Kavya et al., 2023) (Rani et al., 2023).

2.4.5. Cellulase Activity

The cellulase ability of the organism was determined by spot inoculating the isolates on CMC agar plates and flooding with 1% Congo red after incubation for 48 hours. (Timofeeva et al., 2023) (Ogunniyi et al., 2024).

2.5. Seed Germination Assay

Unprimed wheat seeds were surface sterilized with 0.5–1% sodium hypochlorite and 70% ethanol, rinsed with sterile distilled water, and soaked in bacterial suspension ($\sim 10^7$ CFU/mL) for 2 h. Control seeds were soaked in sterile water. 15 seeds were sown per pot containing sterile and non-sterile soil and incubated under room conditions for 15 days. Germination percentage and relative seed germination were calculated using standard formulas. (Oliveira et al., 2017) (Naqqash et al., 2022) (Yasuda et al., 2022).

III. RESULTS AND DISCUSSIONS

3.1. Enrichment and Isolation on Selective Media

Enrichment of soil sample 1, 2, 3 was done in NFB (Nitrogen free Bromothymol blue) Media and *Azospirillum* media. The isolates from the enriched medium were plated onto selective *Azospirillum* medium, which produced transparent, well-isolated colonies after one week of incubation.

3.2. Characterization and Identification of Bacterial Isolates

The bacterial isolates were characterized and identified based on their colonial characteristics and cellular morphology and was identified to be *Azospirillum lipoferum*.

Table 1. colony characteristics of isolates from soil sample 1, 2, 3.

Soil Sample	Isolate from S1	Isolate from S2	Isolate from S3
Size	<1mm	<1mm	1mm
Shape	Circular	Circular	Circular
Colour	Transparent	Transparent	Transparent
Consistency	Mucoid	Mucoid	Mucoid
Margin	Entire	Entire	Entire
Elevation	Flat	Flat	Flat
Gram Nature	Gram Negative	Gram Negative	Gram Negative
Morphology	Rods	Rods	Rods

3.3. Biochemical Characterization of Isolates Obtained from Soil Sample 1,2,3

Table 2. Biochemical characterization of bacterial isolates.

Soil Sample	Catalase	Growth in 3% NaCl	Oxidase	Sugar Fermentation Test				Voges-Proskauer (2% Glucose)
				Mannitol	Glucose	Galactose	Sucrose	
S1	-	+	+	A	A	A	A	-
S2	-	+	+	A	A	A	A	-
S3	-	+	+	A	A	A	A	-

3.4. Determination of IAA (Indole Acetic Acid) Production by the Isolates using Gordon and Weber Method.

Table 3. Standard IAA production.

Tube No	Required Concentration (ppm)	Amount of Stock (ml)	Amount of Diluent (ml)	Total Volume (ml)	Salkowski Reagent (ml)		Absorbance at 540 nm
1	5	0.05	9.95	10	2	Incubate at 25 mins at RT	0.12
2	10	0.1	9.9	10	2		0.13
3	20	0.2	9.8	10	2		0.2
4	50	0.5	9.5	10	2		0.38
5	100	0.1	9	10	2		0.57
6 (Blank)	0	-	10	10	2		0
7. Soil Sample 1	unk	1.0 (Cell-free supernatant)	9	10	2		0.24
8. Soil Sample 2	unk	1.0 (Cell-free supernatant)	9	10	2		0.20
9. Soil Sample 3	unk	1.0 (Cell-free supernatant)	9	10	2		0.22

Table 4. Concentration of isolates from S1, 2, 3 from standard IAA plot.

Sample OD Concentration		Concentration of Unknown from Graph
0.24	Isolate from soil S1	42.37ppm
0.20	Isolate from soil S2	33.71ppm
0.22	Isolate from soil S3	38.04ppm



Fig. 1. Standard dilution tubes of IAA (Indole-3-Acetic acid). Fig. 2. S1,S2, S3 tubes showing pink coloration indicating IAA production.



3.5.1. Phosphate Solubilization Activity of the Isolates (Qualitative Method)

The phosphate solubilizing ability of the isolates from soil sample 1, 2, 3 was determined by spot inoculating the isolates on the plates of pikovaskays agar medium and after incubation Halo formation was observed around the colony from soil sample 1, 2, 3.

Table 5. zone of clearance observed on Pikovakays medium in mm.

Soil Sample	Zone of Clearance in (mm)
S1	15
S2	16
S3	9

3.5.2. Phosphate Solubilization Activity of the Isolates (Quantitative Method)

Table 6. Standard table for phenol.

Tube No	Conc. of Phenol (mcg/ml)	Volume of Stock Phenol (ml)	Volume of Distilled Water (ml)		0.5N NaOH (ml)	0.5N NaH ₂ CO ₃ (ml)	4-Amino Antipyrine (ml)	K ₃ Fe (CN) 6 (ml)	OD at 530 nm
1.	4	1	4	Incubate at 37°C for 2 hrs	0.8	1.2	1.0	1.0	0.19
2.	8	2	3		0.8	1.2	1.0	1.0	0.28
3.	12	3	2		0.8	1.2	1.0	1.0	0.33
4.	16	4	1		0.8	1.2	1.0	1.0	0.58
5.	20	5	–		0.8	1.2	1.0	1.0	0.62
6.	Blank	–	5		0.8	1.2	1.0	1.0	0

Table 7. Assay table for quantitative estimation of phosphate solubilization.

Reagents	Control	Test
Buffered disodium phenyl phosphate	5.0	5.0
Supernatant of broth culture	–	1.0
Uninoculated medium	1.0	–
Incubate at 37°C for 2 hrs		
0.5N NaOH (ml)	8.0	8.0
0.5N NaH ₂ CO ₃ (ml)	1.2	1.2
4-Aminoantipyrine (ml)	1.0	1.0
K ₃ Fe (CN) 6 (ml)	1.0	1.0
Absorbance at 530 nm	0.05	S1 = 0.13, S2 = 0.15, S3 = 0.18

IV. CALCULATION

Absorbance of Test - Absorbance of Control

The absorbance values were calculated using the formula and then the obtained results were plotted on the st-



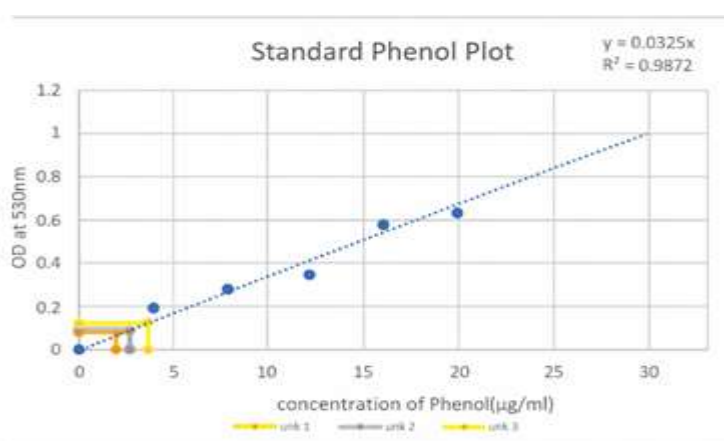
standard graph to determine the final concentration.

Table 8. Values obtained after calculation to be plotted on standard phenol plot.

Isolates from Soil Sample	Values Plotted on Standard Graph (530nm)
S1	0.08
S2	0.10
S3	0.13

Table 9. Concentration of isolate from S1, 2, 3 obtained from graph.

Sample OD at 530nm		Concentration of Unknown from Graph (mcg/ml)
Isolate from soil S1	0.08	2.11
Isolate from S2	0.10	2.73
Isolate from S3	0.13	3.66



Graph 2: Standard phenol plot.



Fig. 3. Standard dilution tubes for quantitative assay for phosphate solubilization.



Fig. 4. Unknown Tubes for S1, S2, S3..

3.6. Cellulase Production

The cellulase producing ability of the organism was determined by a qualitative method after incubation the CMC plates were flooded with 1% congo red and zone of clearance was observed around the colony, indicating its ability to produce cellulase enzyme. Isolates from soil sample 1, 2, 3 showed zone of clearance around the colony.

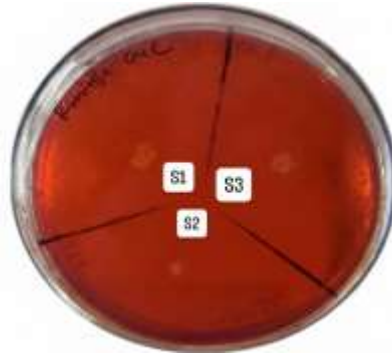


Fig. 5. Zone of clearance seen around the colony on CMC plates.

3.7. Ammonia Production by the Isolates

Ammonia production by the isolate was also determined, and the development of a brown to yellow colour after the addition of Nessler's reagent indicated positive ammonia production.

3.8. HCN Production by the Isolates

The HCN production ability of the isolate was determined using a qualitative assay method, and a change in colour of the indicator paper confirmed the production of hydrogen cyanide.

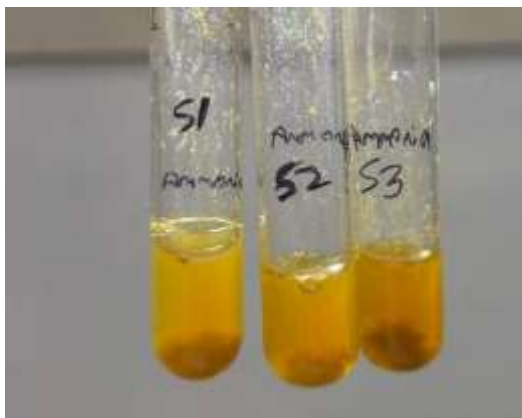


Fig. 6. Development of a brown to yellow colour after the addition of Nessler's reagent

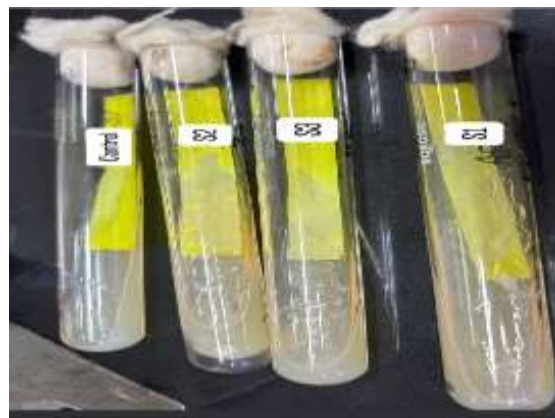


Fig. 7. Tubes of S1, S2, S3 before the production of HCN.



Fig. 8. Tubes of S1, S2, S3 after the production of HCN.



Fig. 9. Indicator paper showing Brown colouration.



Fig. 10. Early plant growth seen in non-sterile soil after 15 days.



Fig. 11. Early plant growth seen in sterile soil after 15 days.



Fig. 12. Root and shoot elongation of isolates from S1, 2, 3 after 15 days.



Fig. 13. Root and shoot elongation of isolates from S1, 2, 3 after 15 days.

Table 10. Root, shoot length and no. of leaves observed after 15 days of growth period.

Isolate from Soil Sample	Total Number of Seeds Planted	Number of Germinated Seeds	Root Length (cm)	Shoot Length (cm)	Number of Leaves
For Non-Sterile Soil (To check effect of Test organism with Native soil organisms)					
S1	15	2	7cm,5cm	25cm, 24cm	5
S2	15	2	18cm,28cm	27cm, 32cm	5
S3	15	4	9cm, 6cm, 7cm, 3cm	21cm, 18cm, 14.5cm, 18cm	12
Control	15	2	1cm, 1cm	1cm, 1cm	3
For Sterile Soil (To check Direct effect of Test organism)					
S1	15	1	5.5cm	18.5cm	3
S2	15	1	6cm	29cm	3
S3	15	1	3cm	17cm	2
Control	15	1	1cm	10.5cm	1



$$\text{Germination Index (\%)} = \frac{\text{NUMBER OF GERMINATED SEEDS}}{\text{TOTAL NUMBER OF SEEDS}} \times 100$$

Table 11. Germination Index of plant in sterile and non-sterile soil.

Seeds Inoculated with Soil Sample	Germination Index %	
	Non-Sterile Soil	Sterile Soil
S1	13.3%	6.6%
S2	13.3%	6.6%
S3	26.6%	6.6%
Control	13.35	6.6%

3.10. Statistical Analysis

The experimental data were analysed using One-Way ANOVA to compare the mean values among isolates S1, S2, and S3. For IAA production, no significant difference was observed among the isolates ($p > 0.05$), indicating similar IAA production levels. However, phosphate solubilisation activity showed a significant difference among the isolates ($p < 0.05$). This indicates that at least one isolate differed significantly in phosphate solubilisation activity. Overall, IAA production was similar among isolates, whereas phosphate solubilisation activity varied significantly.

IV. DISCUSSION

The present study investigated the biochemical characteristics, plant growth promoting (PGP) traits, and seed germination effects of rhizospheric isolates identified as *Azospirillum lipoferum* spp. The results demonstrated that all isolates possessed multiple biochemical and functional traits associated with plant growth promotion, although variations were observed among isolates in their efficiency and plant growth promoting performance. Biochemical characterization showed that the isolates were oxidase positive and capable of growth in 3% NaCl, while catalase and Voges-Proskauer tests were negative. Oxidase positivity indicates the presence of cytochrome-c oxidase and reflects an active respiratory metabolism typical of microaerophilic diazotrophs. The ability to grow in 3% NaCl indicates moderate salt tolerance, suggesting that the isolates may survive in slightly saline agricultural soils. Carbohydrate fermentation tests showed acid production without gas formation for glucose, mannitol, galactose, and sucrose, indicating metabolic versatility and efficient carbohydrate utilization. These biochemical characteristics are consistent with reported metabolic profiles of *Azospirillum* isolates from cereal rhizospheres (Ogunniyi et al., 2024; Sun et al., 2025).

All isolates produced Indole-3-acetic acid (IAA), confirming their phytohormone producing ability. Quantitative estimation showed that isolate S1 produced the highest IAA concentration (42.37 ppm), followed by S3 (38.04 ppm) and S2 (33.71 ppm). IAA production is important because it enhances root elongation, lateral root formation, and root hair development, thereby improving nutrient and water uptake. The variation in IAA production among isolates suggests strain-specific differences in auxin biosynthesis pathways, which is commonly reported in *Azospirillum* strains associated with cereal crops (Naqqash et al., 2022; Ayyaz et al., 2016).

Phosphate solubilization was confirmed by halo zone formation on Pikovskaya's agar and quantitative estimation showed that isolate S3 had the highest phosphate solubilization (3.66 $\mu\text{g/ml}$), followed by S2 (2.73



$\mu\text{g/ml}$) and S1 (2.11 $\mu\text{g/ml}$). The differences among isolates may be due to variation in organic acid production or phosphatase enzyme activity responsible for solubilizing insoluble phosphate. This trait plays an important role in improving phosphorus availability in soil, which is essential for plant growth and development (Mukherjee et al., 2019; Naqqash et al., 2022).

Cellulase activity was observed in all isolates through the formation of a clearance zone on CMC agar, indicating cellulose degradation ability. Although the activity was moderate, cellulase production contributes to organic matter decomposition, nutrient cycling, and improved microbial survival in the rhizosphere. Ammonia production was confirmed by Nessler's reagent test, indicating ammonification activity which contributes to nitrogen availability in the rhizosphere. Hydrogen cyanide (HCN) production was also detected qualitatively, indicating potential biocontrol activity against soil-borne pathogens. The presence of multiple traits such as nitrogen fixation potential, IAA production, phosphate solubilization, ammonia production, cellulase activity, and HCN production indicates the multifunctional nature of the isolates, which is considered important for effective plant growth promotion through multiple synergistic mechanisms (Sun et al., 2025; Timofeeva et al., 2023).

The seed germination and early plant growth assay further confirmed the plant growth promoting potential of the isolates. In non-sterile soil conditions, isolate S3 showed a higher germination index (26.6%) compared to S1 and S2 (13.3%) and the control (13.3%). Root and shoot lengths were also higher in inoculated treatments compared to the control, indicating improved seedling growth. The better performance in non-sterile soil suggests that interaction between the introduced isolates and native soil microflora may enhance nutrient availability and phytohormone production. In sterile soil conditions, the germination index was lower (6.6%) for all treatments including control, although inoculated treatments still showed slightly higher root and shoot growth than control. This indicates that the absence of native microbial communities may reduce the overall plant growth promoting effect, suggesting that microbial interactions in natural soil play an important role in plant growth promotion.

Overall, among the tested isolates, S3 showed the most promising effect on early wheat seedling growth, particularly under non-sterile soil conditions, while S1 showed the highest IAA production. The variation among isolates in IAA production and phosphate solubilization efficiency highlights the importance of strain selection for agricultural applications. The results indicate that these *Azospirillum* isolates possess multiple plant growth promoting traits and have potential for use as biofertilizers in sustainable agriculture.

V. CONCLUSION

The present study confirms that rhizosphere soils from geographically distinct agricultural regions harbor metabolically active diazotrophic bacteria possessing multiple plant growth promoting (PGP) traits of agronomic relevance. The isolated strains exhibited functional diversity, including nitrogen fixation potential, indole-3-acetic acid (IAA) production, phosphate solubilization, ammonia production, hydrogen cyanide (HCN) production, cellulase activity, and moderate salt tolerance. The concurrent expression of these traits demonstrates a multifunctional mode of action, enabling the isolates to enhance nutrient mobilization, modulate root architecture, and potentially contribute to biocontrol activity within the rhizosphere.

Comparative evaluation indicated variability among isolates, particularly in phosphate solubilization efficien-



-cy, where S3 demonstrated significantly higher activity ($p < 0.05$), while IAA production did not differ significantly among isolates. The superior performance of S3 in phosphate solubilization and wheat seed germination assays under non-sterile soil conditions highlights its potential as a promising bioinoculant candidate. The enhanced performance in natural soil further suggests that synergistic interactions with native microflora may amplify plant growth promoting effects.

Overall, the findings underscore the importance of indigenous microbial resources in sustainable agriculture. The identified isolates, particularly S3, exhibit measurable potential for application in biofertilizer development aimed at improving early seedling establishment and nutrient use efficiency in wheat. However, further validation under field conditions and large-scale trials is necessary to confirm their consistency, stability, and agronomic effectiveness.

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