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Evaluation of Extracellular Enzyme Activity of Azotobacter spp. in Relation to Plant Growth-Promotion

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ABSTRACT

Azotobacter spp., a group of free-living nitrogen-fixing bacteria, play a pivotal role in sustainable agriculture by enhancing soil fertility and promoting plant growth through multiple mechanisms. This study investigates the plant growth-promoting traits of Azotobacter isolates, including their ability to secrete extracellular enzymes such as amylase, lipase, protease, cellulase, chitinase, catalase, and ACC deaminase, as well as produce phytohormones, fix atmospheric nitrogen, production of siderophore, and mineralization of Phosphate. Isolates were obtained from the garden soil of the Patan region of Gujarat, India. They were characterized using morphological, microscopical, biochemical, and screening techniques. Enzyme activity was quantified using plate assays, and the enzyme index was calculated to assess their hydrolytic potential. The results demonstrated significant variability among strains, with some exhibiting high enzymatic activity and notable effects. Lastly, one potent isolate, PGY11, was selected for molecular identification and identified as **Azotobacter vinelandii.** These findings underscore the potential of Azotobacter spp. as bioinoculants for improving crop productivity and soil health, particularly under stress conditions.

Keywords: Azotobacter spp., extracellular activity of amylase, lipase, protease, cellulase, chitinase, catalase, and ACC deaminase enzymes, and PGP traits.

1. INTRODUCTION

For understanding the soil bacteria that can sustain soil fertility in the rhizosphere region. It is essential to determine and raise the level of soil fertility and yield production. It will benefit plant growth, and several rhizobacteria genera have been identified as Plant Growth- Promoting Rhizobacteria (PGPR). PGPR is a class of bacteria that is frequently used as a biological fertilizer due to its ability to promote plant growth. One goal of sustainable agriculture is to achieve sufficient yields while preventing soil deterioration. Among other methods for boosting agricultural production, inoculation of PGPR, which enhances plant growth in an environmentally friendly way. PGPRs are distinguished by their capacity for rapid growth, metabolism, and environmental adaptation. Certain PGPR bacterial strains are being used commercially as biofertilizers. These comprise the following genera, including Azotobacter, Bacillus, Burkholderia, Enterobacter, Klebsiella, Pseudomonas, Serratia, Azospirillum, etc. Among them, Azotobacter species

most studied bacteria in plant root growth and development of plants [1].

Beijerinck, a Dutch microbiologist and botanist, made the initial discovery of Azotobacter in 1901 [2]. Azotobacter are aerobic, Gram-negative, free-living, aerobic diazotrophs, spherical or oval-shaped, and 2 to 10 µm long bacteria. Azotobacter uses nitrogen gas from the atmosphere to synthesize its cellular proteins, which contribute to soil fertility by fixing the nitrogen in soil. This bacterium stimulates rhizospheric microorganisms, which improve crop development and production. Azotobacter species have been reported to be sensitive to high temperature (above 35 °C), acidic pH, and high salt. There are seven species in the genus Azotobacter, including Azotobacter crococcum, Azotobacter armeniacus, Azotobacter beijerinckii, Azotobacter vinelandii, Azotobacter paspali, and Azotobacter salinestris [3].

Beyond nitrogen fixation, these bacteria exhibit a rich repertoire of plant-growth-promoting (PGP) traits, including phytohormone production, phosphate solubilization, and the secretion of extracellular hydrolytic enzymes that participate in organic matter decomposition, nutrient mineralization, and direct antagonism of fungal pathogens. These multifunctional attributes render Azotobacter attractive candidates for biofertilizer development and make them valuable components of sustainable agricultural strategies [4].

PGPR can promote plant growth in either a direct or indirect way. The synthesis of phytohormone (auxin, cytokinins, and gibberellins), the decrease in ethylene level, and the enhancement of mineral uptake via boosting root surface area or triggering ion uptake system are the key components of direct plant growth stimulation. Additionally, indirect plant growth stimulation encompasses the development of systemic resistance and the biological control of diseases via the production of HCN or antimicrobial agents, as well as the production of extracellular hydrolytic enzymes [5].

Extracellular enzyme production by Azotobacter influences both soil biochemical cycles and plant health. Lytic enzymes such as chitinase and protease degrade fungal cell walls and proteins, contributing to biocontrol activity, while cellulases/β-glucosidases and pectinases accelerate the breakdown of complex plant residues, releasing C and micronutrients that become available to plants and the microbial community. These enzyme-mediated processes also interact with other PGP mechanisms (e.g., siderophore release, IAA production) and with microbial community dynamics, so that enzyme output can modulate rhizosphere competence and the effectiveness of inoculants under field conditions [6].

Field and pot studies published in 2025 report that inoculation with Azotobacter strains exhibiting measurable cellulase/chitinase/protease activities improved seedling vigor, root architecture, and resilience under contaminated or nutrient-poor soils, indicating a tangible benefit for early-stage plant survival [7]. These Enzyme Activities Matter for Plant Survival in the following way [8]:

- Enhanced Stress Resilience: Catalase and ACC deaminase both reduce oxidative and hormonal stress signals, enabling better root development and plant Vigor.
- Improved Soil Fertility and Nutrient Access: Enzymatic degradation of organic molecules (via cellulase, protease, etc) potentially improves nutrient release and soil structure - creating a more supportive environment for plants.
- Growth Promotion: Lower ethylene levels and improved nutrient availability collectively stimulate better seed germination, root and shoot growth, and overall plant performance.

The goal of the current study was to observe the effect of different enzymes produced by Azotobacter spp. All enzymes directly or indirectly affect plants by promoting plant growth, increasing soil fertility, stress tolerance, and helping to survive against phytopathogens. The bacterial and fungal infections may be controlled by an extracellular hydrolytic enzyme such as amylase, lipase, protease, cellulase, chitinase, catalase, and 1-aminocyclopropane-1- carboxylic acid (ACC) deaminase. By integrating enzyme assays

with functional plant assays, the work will help identify strains with the most promising enzyme-mediated PGP and biocontrol potential for development as biofertilizers/bioprotectants. The role of all enzymes is mentioned in Table 1.

Table 1: Role of all enzymes in plant growth promotion

No.	Enzyme	Soil Function	Plant Survival Benefit	Reference
1	Amylase	Degrade starch	Enhance rhizosphere microbial activity	[10]
2	Lipase	Degrade lipid	Enhance soil nutrient recycling	[11]
3	Protease	Denature protein	Releases nitrogen sources from the plant	[10]
4	Cellulase	Degrade cellulose	Improve soil structure and root access	[9]
5	Chitinase	Degrade the fungal cell wall	Offer biocontrol and triggers ISR	[10]
6	Catalase	Degrade H_2O_2	Reduces oxidative stress in the rhizosphere	[13]
7	ACC Deaminase	Degrade ACC	Improve stress resilience and growth	[12]

2. MATERIALS AND METHODS

2.1 Isolation, Morphological Characterization, and Screening

The isolates were previously effectively isolated using selective media from garden soil of Patan (23.8500° N, 72.1210° E). To identify the cultivated colonies, morphological, microscopical, and biochemical characterizations were performed. Cultivated colonies were examined closely for morphological study by size, shape, margin, elevation, appearance, opacity, and pigmentation [14]. Microscopic features were evaluated by Gram staining and capsule staining. Further biochemical characterization was done by the IMViC test. Various physical parameters, including pH, temperature, and salt, were used for screening purposes [15].

2.2 Evaluation of extracellular hydrolytic enzyme production

Selected bacterial isolates were screened for the production of hydrolytic enzymes, including amylase, lipase, protease, cellulase, chitinase, catalase, and ACC deaminase. For the quantitative data of enzyme production enzyme index was calculated with the following equation.

$$\text{Enzyme index (EI)} = \frac{\text{Colony diameter} + \text{Cleare zone diameter}}{\text{Colony diameter}}$$

2.2.1 Amylase

Evaluation of the amylase-producing bacteria was done by using starch agar medium that contained (g/L) agar 20.0, soluble starch 10.0, beef extract 3.0, and peptone 5.0. After the spot inoculation into starch agar plates, the bacterial isolates were incubated for 24 to 48 hours at 37 °C. Following incubation, 1% iodine solution was flooded onto the plates. Amylase production and starch hydrolysis were confirmed by calculating EI with the development of clear zones surrounding bacterial colonies against a blue background [16].

2.2.2 Lipase

Evaluation of bacteria that produce lipase, by using tributyrin agar medium that contained (g/L) peptone 5.0, NaCl 5.0, agar 20, and tributyrine 10 ml. After the spot inoculation into tributyrin agar plates, the bacterial isolates were incubated for 48 to 72 hours at 37°C. Following incubation, the development of transparent hydrolytic zones surrounding the bacterial colonies suggested production of lipase, and based on that, EI was calculated [17].

2.2.3 Protease

Protease-producing bacteria were evaluated on skimmed milk agar plates that contained 10% skim milk powder and 2% agar. 20 µl of a 72-hour-old culture of each selected bacterial isolate was spotted onto a skimmed milk agar plate and incubated at 28 °C for 48 hours. After the incubation, the colonies on these plates were checked for the formation of a clear zone surrounding the colonies that produced the protease enzyme. Diameter of zone and colonies were used to calculate EI [18].

2.2.4 Cellulase

To determine the cellulase-producing isolates, a CMC minimal agar medium supplemented with 0.1% carboxymethyl cellulose (w/v), 0.1% NaNO3, 0.05% MgSO4, 0.1% K2HPO4, 0.1% KCl, 0.05% yeast extract, and 1.5% Agar was used. 20 µl of a 72-hour-old culture of each selected bacterial isolate was spotted and incubated for 5 days at 28°C. After the incubation, Gram iodine was flooded onto the CMC agar plates, which were then left to remain at room temperature for 10 min. Then all plates were flooded with 1M NaCl solutions and observed for the clear zone surrounding the colonies that produced the cellulase enzyme, and then computed EI [19].

2.2.5 Chitinase

Chitinase-producing bacterial isolates were inoculated into a colloidal chitin agar plate having (g/L) NH4SO4 1, KH2PO4 0.2, K2HPO4 1.6, NaCl 0.1, MgSO4 and FeSO4 0.01, CaCl
0.02, and Agar 20. Incubated all the plates at 28°C for 5 days. The production of distinct zones after incubation served chitinase production. Diameter of zone and colony measured, and EI was calculated [20].

2.2.6 Catalase

The hydrogen peroxide assay was used for the catalase production by isolates. On sterile glass slides, freshly cultured bacteria were smeared, and the addition of a few drops of a 3% hydrogen peroxide solution resulted in the quick and strong evolution of oxygen bubbles, which showed that hydrogen peroxide was breaking down into water and oxygen. The degree of catalase production was qualitatively mirrored in the intensity of bubble formation [21].

2.2.7 ACC deaminase

A minimal salt agar medium containing 1-aminocyclopropane-1-carboxylate (ACC) as the only nitrogen source was used for the production of ACC deaminase. Medium contains (g/L) K_2HPO_4 0.5, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.1, $FeSO_4$ (trace), $CaCl_2$ 0.02, agar 20, and ACC 3 mM (≈ 0.30 g/L). The direct inoculation of the bacterial isolates on media was followed by 48 to 96 hours of incubation at 28 to 30°C. When plates show growth of isolates compared to no nitrogen control plates, it was interpreted as a qualitative measure of ACC deaminase activity [22].

2.3 PGP TRAITS ACTIVITIES OF ISOLATES:

2.3.1 IAA production

To inoculate the isolates, 25 ml of a modified Luria broth medium containing 50 µ/ml tryptophan was

used. They were incubated for a whole day on a shaker at 30°C. The cultures were centrifuged for 15 minutes at 10,000 g. 2-3 drops of orthophosphoric acid were added to 2 ml of supernatant. Once 4 ml of Salkowski reagent has been added and allowed to rest at room temperature for 30 minutes in a dark environment, a pink tint appears, signifying the formation of IAA. We measured the absorbance at 530 nm. The auxin production was computed with a conventional graph [23].

2.3.2 Nitrogen fixation

Nitrogen-free malate agar medium supplemented with BTB dye was used to check the nitrogen fixation ability of isolates. This contains (g/L) sodium malate 5.0, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.2, NaCl 0.2, $CaCl_2$ 0.02, $FeCl_2$ 0.01, $Na_2MoO_4.2H_2O$ 0.002, BTB dye 0.5%, and agar powder 20. Isolates were inoculated by spotting, and plates were incubated for 24 hours at 30 °C. A visible blue coloured zone observed around the colonies indicates that the isolates are nitrogen fixers, and the nitrogen fixation index was calculated by the following equation [24].

$$\text{Nitrogen fixation index (NFI)} = \frac{\text{Colony diameter} + \text{Blue coloured zone diameter}}{\text{Colony diameter}}$$

2.3.3 Siderophore production

The Chrome azurol S agar medium was used to quantify the siderophore production. Test organisms were spot inoculated on Chrome Azur S agar plates after they had been prepared and divided into four equal sectors. The plates were then incubated for 48 to 72 hours at 30°C. It was thought that the development of a yellow-to-orange halo zone around the growth was advantageous for siderophore synthesis. Based on the diameter of zones and colonies, SPI – siderophore production index is calculated with the following equation [25]

$$\text{Siderophore production index (SPI)} = \frac{\text{Colony diameter} + \text{Orange halo zone diameter}}{\text{Colony diameter}}$$

2.3.4 Phosphate solubilization

Pikovskaya media was used in the preparation of the plates. After the cultures were spotted on the plates, they were incubated for seven days at 30 °C. A prominent halo zone near the bacterial growth was detected after incubation, and the phosphate solubilization index was computed using the following formula, which showed a clear zone of solubilization [26].

$$\text{Phosphate solubilization index (SI)} = \frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}}$$

2.4 MOLECULAR CHARACTERIZATION:

For the identification of one potent isolate sent to Bioagro Innovators LLP – Gandhinagar. Molecular characterization has been done by 16s rRNA Sanger sequencing of partial gene sequence with several steps, including isolation of genomic DNA, PCR with 27F &1429R primers, Gel electrophoresis, cycle sequencing PCR, gel purification, run setup through Bio System, and data analysis by a genetic analyzer. The sequence was screened and subjected to the BLAST search program in the National Center for Biotechnology Information (NCBI) to obtain some of the best-matching sequences.

3. OBSERVATIONS AND RESULTS

3.1 Isolation, Morphological, Microscopical, and Biochemical Characterization, and Screening

A total of 16 bacterial isolates were isolated using a specific aseptic technique. Which are named as PAN13, PBND1, PBN13, PCY13, PCN13, PCND3, PDND1, PDND2, PDN11, PEND1, PEN11, PEND3, PFN11, PFYD1, PGND1, and PGY11. All 16 isolates were characterized by morphological, microscopical, and biochemical studies. Morphologically, most of the isolates have a circular shape, convex elevation, entire margin, smooth surface, opaque opacity, and yellowish or brown pigmentation. The data of microscopical characterization, biochemical characterization, and screening by pH, temperature, and salt tolerance assay are mentioned in Table 2. Among them, 7 isolates were screened out based on various stress tolerance parameters, including salt, pH, and temperature, which are PAN13, PBND1, PBN13, PCY13, PCND3, PFYD1, and PGY11. All these screened isolates were selected for further study.

Table 2: Microscopical characterization, Biochemical characterization, and Screening assay by pH, Temperature, and salt tolerance assay.

No.	Isolate name	Gram's staining	Capsule staining	IAA production	MR test	VP test	Citrate utilization	Cell Shape	pH assay	Temp. Assay	Salt assay
1	PAN13	-	+	-	+	-	+	Rod	+	+	-
2	PBND1	-	-	-	-	+	+	Rod	+	-	+
3	PBN13	-	-	+	+	-	-	Rod	+	-	-
4	PCY13	-	+	-	+	+	-	Rod	+	+	+
5	PCN13	-	-	+	-	-	+	Rod	-	-	-
6	PCND3	-	+	-	-	-	-	Rod	+	+	+
7	PDND1	-	-	+	-	+	-	Rod	-	-	-
8	PDND2	-	+	-	+	+	+	Rod	-	+	+
9	PDN11	-	+	-	+	-	-	Rod	-	-	-
10	PEND1	-	-	-	+	+	+	Rod	-	+	-
11	PEN11	-	-	-	-	-	-	Rod	-	-	-
12	PEND3	-	+	+	-	+	+	Rod	-	+	+
13	PFN11	-	-	+	-	+	+	Rod	-	-	-
14	PFYD1	-	+	+	+	-	+	Rod	-	+	+
15	PGND1	-	-	-	+	-	-	Rod	-	-	-
16	PGY11	-	+	+	-	+	+	Rod	+	+	+

3.2 Evaluation of extracellular hydrolytic enzyme production

Hydrolytic enzymes play a crucial role in plant growth promotion and soil health by facilitating nutrient cycling and enhancing the root colonization efficiency of PGPR. These enzymes, such as amylase, lipase, protease, cellulase, chitinase, catalase, and ACC deaminase, contribute to the degradation of complex organic compounds into simpler forms, thereby improving nutrient availability in the rhizosphere. In addition, enzyme production aids in the suppression of phytopathogens through lysis of cell walls and competition for nutrients.

Table 3: Extracellular hydrolytic enzyme assay

No.	Name	Amylase Enzyme Index (EI)	Lipase Enzyme Index (EI)	Protease Enzyme Index (EI)	Cellulase Enzyme Index (EI)	Chitinase Enzyme Index (EI)	Catalase Pro.	ACC Deaminase Enzyme Index
1	PAN13	2.1	0	1.4	1.1	0	+	-
2	PBND1	0	1.3	0	3.0	0	-	-
3	PBN13	1.2	0	2.3	0	1.5	+	-
4	PCY13	2.5	1.0	0	2.7	2.1	+	+
5	PCND3	3.5	0	1.6	0	0	+	+
6	PFYD1	0	2.1	0	1.6	0	-	-
7	PGY11	3.0	2.8	3.0	1.8	1.5	+	+
Total		**05**	**04**	**04**	**05**	**03**	**05**	**03**

The ability of Azotobacter spp. to secrete multiple extracellular hydrolytic enzymes reflects their metabolic versatility and potential to promote plant growth both directly and indirectly. The quantitative plate assays performed in the present study revealed significant variation among the Azotobacter isolates in terms of hydrolytic enzyme production, indicating diverse biochemical potential among them.

A total of seven selected screened isolates were evaluated for the extracellular hydrolytic enzyme production. As shown in Table 3, the hydrolytic enzyme secretions were measured using a plate test, and the enzyme activities were expressed in terms of the enzyme index (EI). Out of seven selected isolates, 05 isolates showed amylase activity, 04 isolates showed lipolytic activity, 04 isolates showed proteolytic activity, 05 isolates showed cellulolytic activity, 03 isolates showed chitinase activity, 05 isolates showed catalytic activity, and 03 isolates showed ACC deaminase activity.

3.3 PGP TRAITS ACTIVITIES OF ISOLATES:

The present study characterizes direct mechanisms including plant growth-promoting traits (PGP traits), viz. Indole Acetic Acid (IAA) production, Nitrogen fixation, Siderophore production, and Solubilization of Phosphate.

3.3.1 IAA PRODUCTION

IAA production was quantified with the use of Salkowski's reagent by the spectrophotometric method. Color development was visible at the highest IAA concentration within a minute and continued to increase in dark conditions for 30 minutes. Therefore, the optical density was measured after 30 minutes. The intensity of the dark reddish pink color development in the tube, checked spectrophotometrically at 530 nm, indicated the strong IAA synthesis by the selected isolates. IAA production in LB broth ranged from 3.6 to 6.72 µg/ ml. Amongst all the isolates, IAA production was higher in the isolate PGY11 and lower in the isolate PBN13. Table 4 describes the data on IAA production.

3.3.2 NITROGEN FIXATION

Nitrogen-free malate medium with BTB indicator plates shows a color change surrounding the colony from greenish-blue to yellow. Some isolates also show growth, but they didn't change the color of the media. NFI of all the isolates was calculated, and the highest nitrogen fixed was by PGY11, and the lowest

nitrogen fixed by PCY13.

3.3.3 SIDEROPHORE PRODUCTION

Siderophore production was observed by the yellow or orange halo zone formation around the colony on the CAS agar medium. The diameter of the colony and zone was measured and calculated for the siderophore production index. Table 4 describes the data on siderophore production. Isolate PAN13 produces the lowest amount of siderophore, and the isolate PFYD1 produces the highest amount of siderophore.

3.3.4 PHOSPHATE SOLUBILIZATION

The efficacies of phosphate solubilization are shown in Table 4. When the zone of solubilization was detected surrounding the isolated colony on modified Pikovskaya agar, then the solubilization index (SI) was calculated with the equation mentioned in the protocol using the colony's diameter and zone. The highest amount of phosphate is solubilized by PGY11, and the lowest amount of phosphate is solubilized by PFYD1.

Table 4: PGP traits characterization

No.	Name	IAA Pro. µg/ml	NFI	Siderophore Pro.	P Sol. Index (SI)
1	PAN13	5.1	2.3	0.3	3
2	PBND1	6.5	1.9	0.6	2.9
3	PBN13	3.6	2.8	0.7	2.6
4	PCY13	4.9	1.6	0.5	3.2
5	PCND3	5.8	3.1	0.8	3.4
6	PFYD1	6.2	2.9	1.2	2
7	PGY11	6.72	3.6	1.1	3.8

3.4 MOLECULAR CHARACTERIZATION:

Based on all the results, isolate PGY11 was selected for the molecular identification. PGY11 is a potent isolate that gives most of the tests positive, which indicates PGY11 is the best plant growth promoter. Molecular identification was done at Bioagro Innovators LLP – Gandhinagar. The accession no is **NR_137421.1**, and the identified species is **Azotobacter vinelandii.**

4 CONCLUSION:

A total of 16 bacterial species were identified in the current study from the soil sample collected from Patan region of Gujarat, India. All isolates were identified morphologically and microscopically using accepted techniques. Out of the 16 isolates, 7 isolates (PAN13, PBND1, PBN13, PCY13, PCND3, PFYD1, and PGY11) have screened according to the data. The extracellular hydrolytic enzyme activity of amylase, lipase, protease, cellulase, chitinase, catalase, and ACC deaminase help in plant growth promotion in either direct or indirect way. The finest PGP features of seven selected isolates characterized which include the ability to produce IAA, nitrogen fixation, Siderophore production, and solubilize phosphate also Every test result is positive in the isolate PGY11. Molecular identification of PGY11 suggest that PGY11 is **Azotobacter vinelandii** with **NR_137421.1** accession number. **Azotobacter vinelandii** can be used as a biofertilizer.

5 FUTURE PROSPECT:

The future of this research on Azotobacter spp. hold significant promise for the future of sustainable agriculture due to their nitrogen-fixing ability, stress tolerance, and plant growth- promoting traits. They are increasingly being explored as eco-friendly biofertilizers and biostimulants.

6 DATA AVAILABILITY STATEMENT:

The authors will make the unfiltered raw datasets used to support the findings of this paper public. An online repository contains raw data. The article includes citations to the repositories.

7 REFERENCE

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