**Exploration of Plant Growth-Promoting Traits of Diazotroph Isolates Obtained from Rhizospheric Soil of North Gujarat Region**

**Abstract**

Rhizospheric diazotrophs play a pivotal role in sustainable agriculture by enhancing nutrient availability and promoting plant growth. The present study was conducted to explore native plant growth-promoting diazotrophic bacteria from the rhizosphere of different plants. 3 isolates (Hb1R 1, WhR 2, and GrR 1) were selected out of 24 as promising based on plant growth-promoting traits such as phosphate (P), potassium (K), and zinc (Zn) solubilization, as well as production of indole acetic acid (IAA) and ammonia (NH3). All three isolates exhibited quantitative P solubilization and NH3 production, ranging from 13.55 ± 1.20 to 300.3 ± 7.78 µg/ml and 1.074 ± 0.15 to 9.584 ± 0.165 mM/ml, respectively. Indole acetic acid (IAA) production of 85.6 ± 0.33 to 156.75 ± 1.62 µg/ml after 96 hours of incubation was demonstrated only by GrR 1. Maximum N fixation of 2.15 ± 0.22 mg/ml was exhibited by Hb1R 1, followed by GrR 1 (1.85 ± 0.1 mg N/ml) and WhR 2 (1.03 ± 0.09 mg N/ml). 16S rRNA gene sequencing identified isolates Hb1R 1, WhR 2, and GrR 1 as *Enterobacter cloacae* subsp. *dissolvens* strain Hb1R1, *Enterobacter* *cloacae* strain GrR1, and *Enterobacter* *cloacae* strain WhR2, respectively. These isolates show promise as bioinoculants for sustainable agriculture, warranting further studies on field application and adaptability to enhance crop productivity.

**Keywords:** Diazotrophs · Biological nitrogen fixation · Rhizospheric soil · Plant Growth-Promoting Traits

1. **INTRODUCTION**

“Nitrogen (N) is an essential nutrient that plays a vital role in plant growth and development. It is a fundamental component of amino acids, proteins, chlorophyll, and nucleic acids and is crucial for various physiological processes. In addition to photosynthesis, nitrogen is vital for protein synthesis, which supports enzyme function and structural integrity in plants” (Wang *et al.,* 2024). “The global nitrogen (cycle has been more heavily altered than any other elemental cycle, primarily through fertilizer use and fossil fuel combustion. The amount of N fertilizers used increased from 11 million tons in 1961 to 119 million tons in 2018, since the world's population depends on them for sustenance. However, crops use only approximately 50% of the nitrogen applied through fertilizers, with the remainder lost to various processes such as volatilization, leaching, and denitrification​. This inefficiency not only limits crop productivity but also leads to significant economic losses for farmers” (Fathi, 2022). “Excessive nitrogen loss poses significant challenges, including environmental issues such as water pollution and greenhouse gas emissions. Excessive nitrogen loss also negatively affects terrestrial ecosystems by altering plant community structure and reducing biodiversity. Certain nitrogen-loving plant species can dominate ecosystems, leading to a decline in species richness and changes in wildlife habitat quality. Additionally, nitrogen loss through denitrification results in the release of nitrous oxide (N2O), a potent greenhouse gas that contributes to climate change. Agriculture is a major source of N2O emissions, accounting for a significant portion of global greenhouse gas emissions” (Pan *et al.,* 2022). These environmental impacts underscore the urgent need to improve nitrogen use efficiency in agriculture. Managing nitrogen efficiently is imperative, necessitating a shift toward sustainable agricultural practices that prioritize biological nitrogen fixation (BNF) over chemical fertilizers.

“Symbiotic and non-symbiotic N fixation (free-living N fixation) are the two primary classes of BNF” ([Gaby](https://www.sciencedirect.com/science/article/pii/S0038071723003231" \l "bib14) *[et al.,](https://www.sciencedirect.com/science/article/pii/S0038071723003231" \l "bib14)* [2018](https://www.sciencedirect.com/science/article/pii/S0038071723003231" \l "bib14)). “Free-living N fixation has been recognized as an important source of N input in most terrestrial ecosystems, particularly those lacking abundant symbiotic N-fixing plants” ([Gupta and Roley, 2023](https://www.sciencedirect.com/science/article/pii/S0038071723003231" \l "bib17)). “Free-living diazotrophs are estimated to account for at least one-third of the global total BNF” ([Davies-Barnard and Friedlingstein, 2020](https://www.sciencedirect.com/science/article/pii/S0038071723003231" \l "bib7)). “Unlike symbiotic diazotrophs, these microorganisms can independently fix N without forming a symbiotic relationship and are more widely distributed across different spatial and temporal scales” ([Smercina et al., 2019](https://www.sciencedirect.com/science/article/pii/S0038071723003231" \l "bib51)). They play vital roles in enhancing plant growth and soil fertility by producing phytohormones, solubilizing essential nutrients, and inducing stress resistance. Harnessing native plant growth-promoting rhizobacteria (PGPR) strains adapted to local environments is essential for effective microbial inoculation and sustainable agriculture (Hakim et al., 2020). “Among the different bacterial genera that have been reported as PGP diazotrophs (*Azospirillum, Agrobacterium, Rhizobium, Enterobacter, Beijerinckia, Klebsiella, Phyllobacterium*)” (Vessey, 2003). PGPRs are directly or indirectly involved in promoting plant growth and development (Ahemad & Kibret, 2014).

Diazotrophs, found in the roots and phyllosphere of various C3 and C4 crops, fix atmospheric nitrogen and enhance soil N uptake by promoting root growth (Li et al., 2019. Beyond nitrogen fixation, they support plant development through multiple mechanisms, including phytohormone production, siderophore and antibiotic release, phosphate and micronutrient solubilization, calcite degradation, and ACC deaminase activity (Hakim et al., 2020). The rhizosphere is a nutrient-rich zone surrounding plant roots, plays a crucial role in crop productivity, soil health, and sustainable agriculture (Bandyopadhyay et al., 2017). Enriched by root exudates, it supports microbial populations 10–100 times higher than in bulk soil (Prasad et al., 2014). Beneficial microbes in this zone, known as plant growth-promoting rhizobacteria (PGPR), enhance plant growth and are gaining attention in sustainable farming. Isolating native strains is vital, as indigenous rhizobacteria are better adapted to local conditions and more effective in promoting plant growth (Dobbelaere et al., 2003).

In conclusion, the excessive use of nitrogen poses multifaceted challenges, ranging from environmental and health issues to disruptions in plant growth and development. Biological nitrogen fixation, particularly through diazotrophs, emerges as a sustainable solution, offering a multitude of benefits in enhancing plant growth, nutrient acquisition, and overall soil fertility. Adopting alternative techniques that prioritize biological nitrogen fixation can mitigate the adverse effects of excessive nitrogen use (Khalid et al., 2004). The present study aimed to isolate diazotrophs, characterize their plant growth-promoting traits, and investigate their impact on plant growth.

1. **MATERIALS AND METHODS**
2. Top of Form

**Collection of rhizospheric soil samples and isolation of diazotrophic bacteria**

Rhizospheric soil samples were collected from wheat, pearl millet, groundnut, vegetables (cabbage, ladyfinger, and eggplant), agroforestry (mango, papaya, watermelon, muskmelon, and sugarcane), holy basil, and curry tree rhizosphere from various regions of Banas Kantha, north Gujarat. Soil samples were stored at 4 ˚C for further analysis. Soil samples were serially diluted, and 100 μl of the sample was spread on Ashby’s Mannitol agar medium plates and incubated at 30 ˚C for 3 days. After incubation, purified colonies were selected and preserved on nutrient agar slant (Kumar et al., 2014).

***In vitro* characterization of plant growth-promoting attributes**

**Phosphate solubilization**

To determine the phosphate-solubilizing ability of diazotroph isolates, Pikovskaya’s agar medium containing calcium phosphate as an inorganic source of phosphate was used. Loopful culture was spot inoculated on Pikovskaya’s agar plates. The plates were incubated at 30 ˚C for seven days. The plates were observed for the zone of clearance around the bacterial colony, which indicated solubilization of P. Phosphate solubilizing ability was described as phosphate solubilization index (PSI): the ratio of the colony + halo zone diameter and the colony diameter (Pande et al., 2017).

**Quantitative estimation of phosphate solubilization**

Quantitative analysis of phosphate solubilization of selected isolates was performed in which 1 ml of bacterial culture (1 O.D. at 600 nm) was inoculated in a flask containing 50 ml of sterile Pikovskaya’s broth medium containing calcium phosphate as an inorganic source of phosphate. Flasks were incubated at 30 ± 2 ˚C for 6 days. five ml of samples were collected after 48, 96, and 144 hours. Samples were centrifuged at 6000 rpm for ten minutes, and the supernatant was used to determine the concentration of phosphate. 300 µl of supernatant was taken in the tube, and the final volume was made up to 1 ml using 700 µl deionized water, then 2 ml of reagent B was added. The final volume was made up to 12 ml using deionized water. Tubes were incubated in the dark for 10 minutes. After incubation absorbance of blue color developed was measured at 660 nm using UV UV-Visible spectrophotometer (Thermo Fisher Scientific, Evolution 201, USA). The concentration of phosphate was estimated from the standard graph of phosphate was prepared by plotting the concentration of KH2PO4 on the X-axis and O. D at the Y-axis (Sengupta et al., 2020).

**Potassium solubilization**

For determining the potassiumsolubilization ability, Aleksandrow agar medium containing potassium alumino silicate as an insoluble potassium source was spot inoculated with a loopful culture of isolates. The plates were incubated at 30 ± 2 ˚C for seven days. A clear zone around the bacterial colonies indicated potassium solubilization(Yaghoubi Khanghahi et al., 2018). The potassium solubilization index (KSI) was calculated as described above in PSI.

**Zinc** **solubilization**

Screening of zinc solubilization efficiency was carried out on zinc solubilizing agar medium containing zinc oxide as an insoluble zinc source. Loopful culture was spot inoculated on zinc solubilizing agar plates and plates were incubated at 30 ± 2 ˚C for seven days. A clear zone around the bacterial colonies indicated zinc solubilization (Gandhi & Muralidharan, 2016). The zinc solubilization index (ZSI) was calculated as above in PSI.

**Indole acetic acid production**

All the isolates were screened for their ability to produce indole acetic acid by the method described byAshour et al. (2022) with a few modifications. A loopful of the test culture was aseptically inoculated into a sterile soybean-casein digest medium supplemented with 1% tryptophan, contained in individual test tubes. The tubes were incubated at **30 ± 2 °C** for **three days** under static conditions. Following incubation, **2 mL of Salkowski reagent** (prepared by mixing 2% of 0.5 M ferric chloride in 35% perchloric acid) was added to each tube. The tubes were then incubated in the dark for **10 minutes**. The development of a **pink to red coloration** indicated a **positive reaction for indole-3-acetic acid (IAA) production**.

**Quantitative estimation of IAA production**

Briefly, 1 mL of bacterial culture (adjusted to an OD₆₀₀ of 1.0) was inoculated into a 50 mL flask containing sterile soybean-casein digest medium supplemented with 1% tryptophan. The flasks were incubated at 30 ± 2 °C for four days under static conditions. Samples (2 mL) were collected at 24, 48, and 96 hours, centrifuged at 6000 rpm for 10 minutes, and the supernatant was used for indole-3-acetic acid (IAA) quantification. For IAA estimation, 40 µL of supernatant was mixed with 960 µL of distilled water to make a final volume of 1 mL, followed by the addition of 2 mL of Salkowski reagent. The tubes were incubated in the dark for 10 minutes, and the absorbance of the developed red color was measured at 535 nm using a UV-Visible spectrophotometer (Thermo Fisher Scientific, Evolution 201, USA). The concentration of IAA was estimated from the standard graph of IAA prepared by plotting the concentration of IAA on the X-axis and O. D at the Y-axis (Ashour et al., 2022).

**Ammonia production**

All the isolates were screened for the production of ammonia by the method described by Prasad et al. (Prasad et al., 2014) with minor modifications.A loopful of culture was inoculated individually into sterile peptone nitrate broth in a test tube. Tubes were incubated at 30 ± 2 ˚C for three days. After incubation, 1 ml of Nessler’s reagent (Himedia) was added. The formation of yellow to brown precipitate was considered positive for ammonia production.

**Quantitative estimation of ammonia production**

1 ml of bacterial culture (1 O.D. at 600 nm) was inoculated in a flask containing 50 ml of sterile peptone nitrate broth. 5 ml of sample was collected after 24, 48, and 96 hours. Samples were centrifuged at 6000 rpm for ten minutes, and the supernatant was used to determine the concentration of ammonia. 100 µl of supernatant was taken in a tube, and the final volume was made up to 1 ml using 900 µl of distilled water. Then, one ml of Nessler’s reagent was added. The final volume was made up to eight ml. The absorbance of the yellow to brown color developed was measured at 450 nm using UV-Visible spectrophotometer (Thermo Fisher Scientific, Evolution 201, USA). The concentration of ammonia was estimated from the standard graph of ammonium sulphate prepared by plotting the concentration of ammonium sulphate on the X-axis and O. D at the Y-axis (Abdelwahed et al., 2022).

**Determination of nitrogen-fixing capacity**

The nitrogen-fixing capacity of isolates was determined by the micro Kjeldahl method described by (Kaviyarasan et al. 2020) with slight modifications. One ml of bacterial culture (1 O.D. at 600 nm) was inoculated in sterile Burk’s medium and incubated at 30 ± 2 ˚C for seven days. After the incubation, 50 ml of broth was digested with five g of digestion salt mixture (50:10:01 ratio of K2SO4, CuSO4, and Metallic Selenium) and ten ml of concentrated H2SO4. After digestion, tubes were allowed to cool, and the final volume was made up to 100 ml. For the distillation process, ten ml of each digested sample and 40 percent NaOH were added into the tube and the tube to the apparatus (KEL PLUS DISTYL-EM). In a conical flask, ten ml of two percent boric acid reagent and three to four drops of the mixed indicator were added, and the flask at the receiving end. After nine minutes, the content of the conical flask was titrated with standard 0.1 N H2SO4. The flask containing ten ml of distilled water and ten ml of 40 percent NaOH was used as the blank titer. Percent nitrogen was calculated using the following formula: -

**Abiotic stress tolerance assay**

**Salt tolerance**

NaCl tolerance assay was carried out by the method described by (Akhter et al. 2012) with a few modifications. Five flasks of 50 ml of nutrient broth with 0.5 percent, 2 percent, four percent, 6 percent, and 8 percent NaCl concentrations were prepared. 1 ml of bacterial culture (1 O.D. at 600 nm) was inoculated in all the flasks and incubated at 30 ± 2 ˚C for 72 hours at 120 RPM. The sample was collected after 72 hours, and the optical density was measured at 600 nm using UV UV-Visible spectrophotometer (Thermo Fisher Scientific, Evolution 201, USA). The graph was prepared by plotting optical density on the Y-axis and time on the X-axis.

**Temperature tolerance**

The temperature tolerance assay was done by the method described byGetahun et al. (2020) with minor modifications. Isolateswere streakedon three sets of nutrient agar slants. One set was placed in the refrigerator at 4 ˚C, the second set was placed at 37 ± 2 ˚C, and the third set was placed at 45 ± 2 ˚C. Presence or absence of growth on slants was observed after 48 hours.

**Morphological, cultural, and biochemical characterization of isolates**

Selective isolates were characterization was done based on the color, shape, size, diameter, margin, and texture of the colony on solid nitrogen-free Ashby’s mannitol agar medium and gram staining. Biochemical characterization was done by performing various tests such as catalase test, oxidase test, IMViC test, urease test, carbohydrate fermentation test *viz.,* lactose, mannose, maltose, sucrose, xylose, and arabinose (Cappuccino JG & Sherman N, 1992).

**Molecular identification of potential isolates**

Molecular identification of the most promising diazotroph isolates was performed by 16S rRNA sequence analysis from Eurofins Genomics India Pvt. Ltd., Bengaluru, India. The obtained partial gene sequences were uploaded to the National Center for Biotechnology Information (NCBI), and the highest similarity with the database was checked using the Basic Local Alignment Search Tool (BLAST). The isolates' partial 16S rRNA gene sequences were submitted at NCBI GenBank through BankIt, and the accession numbers were obtained.

**Data analysis**

All the experiments were performed in triplicate, and the data are presented as mean± standard deviation (SD).

1. **RESULTS AND DISCUSSION**

Numerous studies have been conducted on Plant growth-promoting Rhizobacteria (PGPR) across various crops to explore their potential as a sustainable and environmentally friendly substitute for chemical fertilizers. To date, several significant PGPR isolates have been discovered, belonging to diverse genera such as *Agrobacterium, Arthrobacter, Azotobacter, Bacillus, Enterobacter, Pantoea,* and *Rhizobium.* The variation in the number of isolates obtained might be due to several factors, *viz.* root morphology, the stage of plant growth, root exudates, and the physical and chemical properties of the soil, which are reported to influence the occurrence and distribution of microbial communities in the soil and rhizosphere (Mekonnen & Kibret, 2021).

**Collection of rhizospheric soil samples and isolation of diazotrophic bacteria**

In this study, isolation of diazotrophic bacteria was performed from 15 different plant rhizospheric soil samples collected from various locations using standard laboratory procedures. This resulted in the isolation of a total of 24 diazotrophic bacteria in pure culture. The pure culture of bacteria was maintained on NA slants at 4 ˚C for further use (Table 1).

***In vitro* characterization of plant growth-promoting attributes**

Screening results for multiple PGP traits of isolates are given in Table 2. In this qualitative study, out of 24 isolates tested, 3 (12.5%) isolates were able to form a clear zone on Pikovskaya’s agar medium, indicating phosphate-solubilizing activity. A maximum phosphate solubilizing index was observed in isolate Hb1R 1 (2.42 ± 0.083). Phosphorus is one of the major essential macronutrients of plants, which regulates protein synthesis and plays an important role in biological development. The halo zone formation around the bacterial colonies could be due to the production of organic acids or polysaccharides (Paul & Sinha, 2017). (Li et al., 2017) reported similar results with PSI in the range of 2.03 ± 0.02 to 7.64 ± 0.62. In another study, El-Saied et al. [32] and (Singh M, 2018) recorded a phosphate solubilization index of 3.06 and 3.22 in *Enterobacter cloacae* BAU3 and *Enterobacter cloacae* PSB6, respectively.

Of 24 isolates tested, 7 (29%) isolates were able to form a clear zone on the Aleksandrow agar medium, indicating potassium solubilizing activity. The potassium solubilization index ranged from 3.64 ± 0.31 to 2.20 ± 0.08. The maximum KSI of 3.64 ± 0.31 was observed in isolate GrR 1, whereas the lowest KSI of 2.20 ± 0.08 was displayed by EgR 1 isolate. Potassium is one of the vital nutrients that play a key role in plant growth, metabolism, and development, apart from increasing resistance against diseases, pests, and abiotic stresses. Moreover, it helps in activating over 80 different enzymes in plants that contribute towards various plant processes (Etesami et al., 2017). The result of the present study corroborates the finding of El-Saied et al. [32]. In contrast, Yaghoubi et al. (2018) reported KSI ranged from 1.10 ± 0.011 to 1.64 ± 0.089 from isolates of rice rhizosphere.

In the present study, out of 24 isolates tested, 8 (33.33%) isolates showed zinc solubilizing activity. The ZSI ranged from 3.53 ± 0.19 to 2.18 ± 0.02. The maximum ZSI of 3.53 ± 0.19 was exhibited by isolate GrR 1 whereas the lowest ZSI of 2.18 ± 0.02 was determined in SuR 1 isolate (Fig. 1). Zinc is also one of the essential micronutrients and zinc deficiency has been reported as the most common micro nutritional disorder in plants and acts as a major constraint for successful crop production Gandhi and Muralidharan (2016). Gandhi and Muralidharan (2016) isolated zinc-solubilizing bacteria from rhizospheric soil with the highest ZSI of 13.18 ± 1.69.

The development of a pink-to-red color after the addition of the Salkowski reagent was considered positive for IAA production. Out of 24 isolates, 5 (20.83%) isolates (MaR 1, CuR 1, EgR 2, GrR 1, and GrR 2) were able to produce IAA. The phytohormone indole acetic acid is known to promote plant development in terms of root and stem length. Tryptophan is a precursor needed by the majority of plant growth-promoting bacteria for the production of IAA. Root exudates of many plants contain rich supplies of tryptophan, which PGPR utilize for manufacturing and releasing auxins as secondary metabolites in the soil (Mir et al., 2022).

Ammonia production is considered an important trait of PGPR that influences plant growth either directly or indirectly. Production of ammonia by the microbial cells can satisfy the demand for nitrogen by the host plants and promote root and shoot elongation and their biomass. When produced in excess, it can also provide defense against phytopathogens (Panchami et al., 2020). (Kifle & Laing, 2016) tested 92 isolates found from maize and wheat rhizosphere for ammonia production, and all isolates were able to produce ammonia. In this study, the production of ammonia was determined by adding Nessler’s reagent to inoculated peptone nitrate broth. The formation of yellow to brown precipitate was considered positive for ammonia production. Out of 24 isolates, 11 (45.83%) isolates were found positive.

**Table 1. Plant source and location, isolate number and code from different rhizospheric soil samples**

|  |  |  |  |
| --- | --- | --- | --- |
| **Sr. No.** | **Plants and location of the soil sample** | **No. of isolated diazotrophs** | **Code for diazotrophs** |
| 1. | Holy basil rhizosphere 1 - Palanpur | 02 | Hb1R 1-2 |
| 2. | Holy basil rhizosphere 2 - Palanpur | 01 | Hb2R 1 |
| 3. | Mango rhizosphere – Palanpur | 02 | MaR 1-2 |
| 4. | Papaya rhizosphere – Palanpur | 01 | PaR 1 |
| 5. | Muskmelon rhizosphere - Palanpur | 01 | MuR 1 |
| 6. | Curry tree rhizosphere - Palanpur | 01 | CuR 1 |
| 7. | Eggplant rhizosphere - Rampura village | 02 | EgR 1-2 |
| 8. | Wheat rhizosphere - Rampura village | 02 | WhR 1-2 |
| 9. | Pearl millet rhizosphere - Rampura village | 01 | PeR 1 |
| 10. | Cabbage rhizosphere - Rampura village | 02 | CaR 1-2 |
| 11. | Ladyfinger rhizosphere - Rampura village | 01 | LaR 1 |
| 12. | Watermelon rhizosphere - Rampura village | 02 | WaR 1-2 |
| 13. | Pearl millet rhizosphere – Lodpa village | 02 | PeR 1-2 |
| 14. | Groundnut rhizosphere - Nandotra village | 02 | GrR 1-2 |
| 15. | Sugarcane rhizosphere - Rampura village | 02 | SuR 1-2 |
| **Total** | | **24** |  |

**Quantitative estimation of PGP traits**

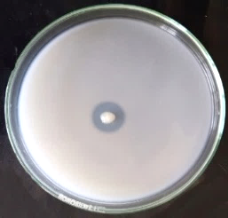
**Phosphate solubilization**

Based on the qualitative assay of PGP traits, three isolates were selected for further study. Phosphate solubilizing activity was quantitatively assessed for each of the three isolates. The isolates exhibited varied efficacies for the solubilization of inorganic P source, which ranged from 13.55 ± 1.20 to 300.3 ± 7.78 µg/ml. Isolate Hb1R 1showed maximum phosphate solubilization (156.5 ± 1.69 µg/ml) after 48 hours. After 96 hours, the WhR 2 isolate exhibited the highest phosphate solubilization activity (218.4 ± 5.02 µg/ml). For the isolate GrR 1, maximum solubilization (300.3 ± 7.78 µg/ml) was observed after 96 hours (Fig. 3). Phosphate concentration reduced after 144 hours, which can be due to the utilization of phosphate for growth. Sengupta et al. (2020) reported solubilization of inorganic phosphate, which ranged from 309.72 to 615.28 µg/ml. In a similar study, Mir et al. (2022) observed phosphate solubilization ranged from 36.69 ± 1.63 to 312.4 ± 1.15 µg/ml.

c

b

a



**Fig 1**: PGP traits of diazotroph isolates. (a). Phosphate solubilization (b). Potassium solubilization, and (c). Zinc solubilization.

**IAA production**

Out of three selected isolates, only isolate GrR1, which showed qualitative indole acetic acid production, was further screened for quantitative analysis of IAA. GrR1 isolate produced IAA ranged from 85.6 ± 0.33 to 156.75 ± 1.62 µg/ml. The highest concentration of IAA was observed after 96 hours. Numerous *Enterobacter* spp. having the potential to produce indole acetic acid (IAA) such as *E. cloacae* H3*, E. cloacae* NII-0931, *E. cloacae* MSR1, *E. cloacae* UW 5*, E.* *asburiae, E. cancerogenus* (Widowati et al., 2019). In contrast to the present study, Kumari et al. [39] reported production of IAA in the range of 45.66–111.94 µg/ml after 48 hours from the medium containing 0.1 percent DL-tryptophan. Similarly, (Jain et al., 2021) reported IAA production within the range of 54.5-6000 µg/ml by the bacterial strains isolated from rhizospheric soil. IAA production is driven by several factors, like culture conditions, growth stage, or substrate availability in the culture medium (Sridevi M & Mallaiah K. V, 2007).

**Ammonia production**

Production of ammonia was found within the range of 1.074 ± 0.22 to 9.584 ± 0.165 mM/ml, in which isolate GrR 1 showed maximum production after 72 hours. A related study identified the highest ammonia production at 371 µM(Abdelwahed et al., 2022). in a similar study, maximum production of 6.51 µmol/ml was found after 144 h (Gohil et al., 2022). Further details are given in Figure 3.

**Fig 2.**  Quantitative analysis of phosphate phosphate-solubilizing ability of selected diazotroph isolates

**Determination of nitrogen-fixing capacity of diazotroph isolates**

The nitrogen-fixing capacity of isolates was determined by the microkjeldahl method. After 7 days, maximum N fixation of 2.15 ± 0.07 mg nitrogen per ml was exhibited by isolate Hb1R 1, followed by GrR 1 (1.85 ± 0.1 mg N/ml) and WhR 2 (1.03 ± 0.09 mg N/ml). Nitrogen is one of the primary elements required for plant development. Since it is a crucial component of proteins, nucleic acids, and other cellular constituents. In contrast to the present study, Kaviyarasan et al. (2020) reported higher N2 fixation in the range from 6.58 to 14.86 mg N/ml after 10 days. *E. cloacae* AKS7 strain was reported to fix 12 mg/l atmospheric nitrogen in 8 days (Chakraborty et al., 2019).

**Abiotic stress tolerance assay**

Based on PGP activity, three isolates were assayed for NaCl tolerance in the presence of various concentrations of NaCl (0.50-8%). All isolates were able to survive at various concentrations of NaCl, in which isolate Hb1R 1 showed an increase in growth at concentrations of 4% and 6% after 72 Hours. Isolate WhR 2 only showed an increase at 4%, and isolate GrR 1 showed a gradual decrease at all concentrations after 72 Hours. Soil bacteria inhabiting salty and arid ecosystems have the potential to promote plant growth under salinity conditions (Mapelli et al., 2013).

Similarly, for the temperature tolerance assay, visible growth on the slant after 48 hours was considered positive, and the absence of growth was considered a negative result. All isolates were able to grow at 4 ˚C and 37 ˚C. No growth was observed at 45 ˚C. The results of this study were found to be similar to Getahun et al. (2020).In this study, we found that isolates were able to grow at 4˚C and 37˚C. While *in vitro* temperature selection may not be seen as a viable strategy for field applications, the ability to tolerate high temperatures can aid in the isolation of competitive plant growth-promoting rhizobacteria (PGPR) amidst fluctuating field temperatures(Patel, 2001).

**Fig 3.**  Quantitative analysis of ammonia production of selected diazotroph isolates

**Table 2. Screening of diazotroph isolates for plant growth-promoting traits**



+ indicate positive result - indicate negative result

**Identification of diazotroph isolates**

All three diazotroph isolates were identified by phenotypic and molecular methods.

**Phenotypic identification**

The primary identification of the selected three isolates was performed by cultural, morphological, and biochemical characteristics. The cultural characteristics of the isolates were determined on Ashby’s mannitol agar plate. The colonies of all the isolates were non-pigmented, medium-sized, opaque, mucoid, creamy, raised, round, and white. Gram’s stained culture smears under a microscope revealed all three isolates as gram-negative short rods. The results of various biochemical characteristics of isolates are depicted in Table 3.

**Molecular identification**

In the present study, a consensus sequence of the 16S rRNA gene was generated from forward and reverse sequence data using codon code aligner software version 5.1.5. The obtained partial gene sequence of three isolates Hb1R 1, WhR 2, and GrR1, respectively, were uploaded to the National Center for Biotechnology Information (NCBI), and the highest similarity with the database was checked using the Basic Local Alignment Search Tool (BLAST). The 16S rRNA partial gene sequence BLAST analysis of three isolates Hb1R 1, WhR 2, and GrR 1 were identified as *Enterobacter cloacae subsp. dissolvens* strain Hb1R1, *Enterobacter cloacae* strain GrR1, *Enterobacter cloacae* strain WhR2, respectively. The phylogenetic tree was built using the neighbor joining method of isolates Hb1R 1, WhR 2, and GrR 1 is depicted in Figure 4, respectively. The partial 16S rRNA gene sequence of the three isolates Hb1R 1, WhR 2, and GrR 1 were submitted to NCBI GenBank with the accession numbers OR258986, OR259056, and OR259059, respectively. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for several reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution), and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Panigrahi et al., 2020). Similarly, Panigrahi et al. (2020) and (J. Gupta et al., 2022) also identified plant growth-promoting bacteria as *Enterobacter cloacae* MG00145 and *Enterobacter cloacae* PNE2, respectively.

**Table 3. Biochemical characterization of isolates**

|  |  |  |  |
| --- | --- | --- | --- |
| Test | Isolate code | | |
| Hb1R 1 | WhR 2 | GrR 1 |
| Catalase | +ve | +ve | +ve |
| Oxidase | +ve | +ve | +ve |
| Indole production | - ve | - ve | -ve |
| Methyl red | - ve | - ve | - ve |
| Voges – Proskauer | +ve | +ve | +ve |
| Citrate utilization | +ve | +ve | +ve |
| Urease | +ve | +ve | +ve |
| Carbohydrate utilization | | | |
| Lactose | - ve | +ve | - ve |
| Mannose | + ve | +ve | + ve |
| Maltose | + ve | + ve | + ve |
| Sucrose | + ve | + ve | + ve |
| Xylose | + ve | + ve | + ve |
| Arabinose | + ve | + ve | - ve |



**Fig 4.** Phylogenetic tree of isolates based on 16S rRNA partial gene sequence made with neighbor neighbor-joining method. (A) phylogenetic tree of bacterial isolate Hb1R 1, (B) phylogenetic tree of bacterial isolate WhR 2, (C) phylogenetic tree of bacterial isolate GrR 1

1. **CONCLUSION**

The findings underscore the role of these microorganisms in enhancing nutrient availability and promoting sustainable agricultural practices. In which three diazotrophic bacteria, *Enterobacter* *cloacae* subsp*. dissolvens* strain Hb1R1, *Enterobacter cloacae* strain GrR1, and *Enterobacter cloacae* strain WhR2, isolated from rhizospheric soil, showed multiple plant growth-promoting traits *in vivo* and *in vitro*. For the potential use of these diazotrophs as bioinoculants, future research should focus on pot studies and field trials, and the ecological adaptability of these isolates to validate their effectiveness under diverse environmental conditions and cropping systems.

## Disclaimer (Artificial intelligence)

## Option 1:

## Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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## Details of the AI usage are given below:

## 1.

## 2.

## 3.

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