# MOLECULAR IDENTIFICATION OF STRESS TOLERANT DIAZOTROPHIC ENDOPHYTIC BACTERIA FROM KALADANI RICE OF JHARKHAND

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**Abstract.**
Nitrogen-fixing endophytic bacteria have been regarded as a potential substitute for chemical nitrogen fertilizers to enhance plant growth and yield. The objective of this study was to isolate and identify endophytic bacteria with diazotrophic properties and abiotic stress tolerance ability from root and stem of kaladana rice grown in Jharkhand. To achieve this goal, endophytic bacteria were extracted from the roots, stems of the plant tissues, and strains that promote plant growth were specifically identified. A total of 11 endophytic bacteria were isolated from the stems and 10 endophytes as diazotrophic nitrogen-fixing bacteria grown in JNFb- media. Further, 4 Out of the isolated bacteria were identified as diazotrophic bacteria using a specific primer set targeting the nifH gene. By performing 16S rDNA sequence analysis, the presence of nifH genes confirmed the existence of four distinct species, namely *Bacillus aryabhatti* (accession no. OQ426536). *Priestia aryabhatti* (accession no. OQ422565). *Bacillus magaterium* (accession no. OQ422490). *Staphycoccus hominis* (accession no. OQ422489). In addition, the study also investigated the phosphate solubilizing and abiotic stress tolerance activity of the isolated bacteria to explore potential mechanisms for plant growth promotion. Out of the four isolates tested, all four strains exhibited significant levels of phosphate-solubilizing and abiotic tolerance activity. The findings of this study strongly indicate that the identified endophytic diazotrophic bacteria have great potential for effectively enhancing plant growth in sustainable agriculture.

**Keywords:** Diazotrophs, Abiotic stress, Endophytes, PGPR, Rice and Phosphate solubilization.

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## Introduction
The rapid growth of the global population is a cause for concern, with estimates projecting it to reach 9.2 billion by 2050. Consequently, there is a pressing need to increase global agricultural production by 60-70% compared to current levels, In order to address the increasing food demand in the next three decades [1]. Traditionally, agricultural production has heavily relied upon commercial fertilizers and pesticides to maximize yields. However, due to increasing worries about the detrimental to mitigate the adverse effects of synthetic agrochemicals on environment and human health, farmers are encouraged to explore more environmentally friendly alternatives [2]. Excessive use of synthetic agrochemicals can also lead to the development of plant pathogen resistance resistance, necessitating the search for alternative approaches [3]. Biofertilizers have emerged as a preferable alternative to synthetic chemicals for improving plant growth plant. The market size of these products has exceeded 4 billion US dollars and is experiencing an annual growth rate of 5-10% [4]. In India, where freshwater sources are heavily Contaminated with nitrogen and phosphorus derived from intensive agricultural practices, bioinoculants have become especially important in addressing the urgent issue of eutrophication [5]. Endophytic micro-organisms, these elements, present within diverse plant tissues, play a vital role in overall plant development and the ability to adapt to both biotic and abiotic stresses [6]. These endophytic bacteria exploit the internal plant environment as a distinct ecological niche, protecting against environmental changes. The mutualistic association between endophytes and their host plants has evolved over millions of years [7-8]. Endophytes occupy the identical ecological niche as pathogens and fulfill the role of biocontrol agents, effectively combating plant diseases [9-11]. Through various mechanisms, endophytic bacteria exert direct and indirect beneficial effects on plants [12] These microorganisms assist plants in acquiring minerals, combatting plant pathogens, and increasing drought and salinity tolerance [13-14]. The plant growth-promoting effects of plant growth-promoting rhizobacteria (PGPR) are attributed to several key mechanisms. Endophytic bacteria contribute to plant growth through various mechanisms, including the

synthesis of plant growth hormones, ACC deaminase activity, production of organic acids, secretion of siderophores, nitrogen fixation, and phosphate solubilization [15]. bacteria and plant growth-promoting rhizobacteria (PGPR) exhibiting traits associated with plant growth promotion and disease resistance. The production of enzymes, hormones, antimicrobial secondary metabolites, and defense-related compounds is primarily responsible for these beneficial effects [16-17]. Moreover, plant-associated endophytic bacteria have been extensively studied for their role in enhancing plant tolerance to abiotic stresses [18–20]. Numerous endophytic bacteria isolated from diverse host plants have demonstrated the capability to enhance salt and drought tolerance in the plants they are inoculated in [21–23]. During recent years, Bacillus species have garnered significant attention due to their potential as synthesis of plant growth-promoting substances, bioactive compounds and secondary metabolites [24-25]. The capacity of Bacillus species to form endospores is highly valuable for agricultural applications, as it allows for longer shelf life and resistance to desiccation and heat exposure. Furthermore, Due to their positive effects on plant growth and their ability to enhance fungal disease resistance, Bacillus-based products are extensively employed as biofertilizers and biocontrol agents in sustainable agriculture [26–28]. To discover new strains with multiple beneficial effects on plant development, research efforts should concentrate on exploring diverse sources of soil and plant hosts, in addition to the existing plant growth-promoting rhizobacteria (PGPR) and endophytes. Medicinal plants, which are well-known for their therapeutic properties, offer a promising avenue for such exploration [29-30]. Therefore, there is a need for the isolation and characterization of more potent and efficient plant growth-promoting rhizobacteria (PGPR) and endophytes Rice (Oryza sativa) belongs to the Poaceae family and is cultivated worldwide as a staple food source [31]. Studies have shown that the continuous cropping of rice can lead to alterations in the agrochemical properties of the soil and a decline in microbial functional diversity [32-33]. Under continuous monoculture, the rhizosphere of rice undergoes changes in both microbial composition and functional diversity. [34]. Recent studies have uncovered that plants have the ability to shape their rhizosphere microbiome, exerting an influence on the diversity and structure of the microbial community [35] Recognizing the potential importance of rice production, we conducted a study focused on isolating and identifying diazotrophic endophytic bacteria from the roots and stems of rice, as well as examined their nitrogen fixing and phosphate utilization characteristics.

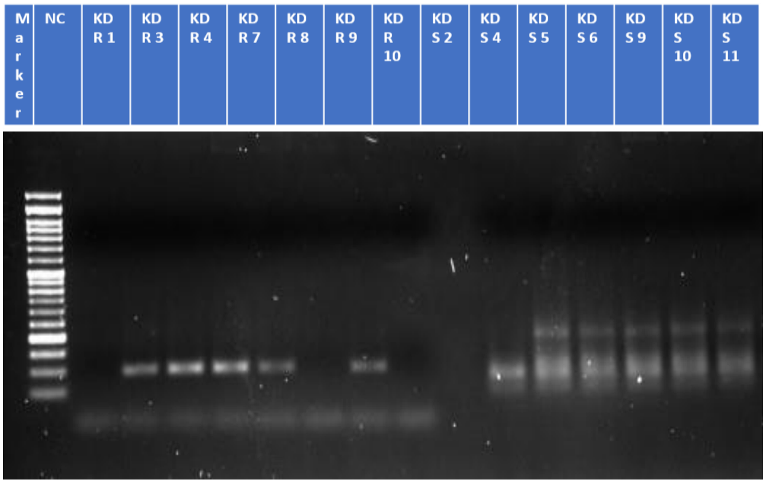
**Materials and Methods**

Isolation of Endophytic Bacteria from Rice Roots and Stems During the winter of 2021, samples of rice were collected in the Ranchi District (23°22′12.18″N, 85°19′30.14″E) in the Indian state of Jharkhand. The roots and stems of the rice plants were taken, the endophytic bacteria were isolated from the roots and stems after undergoing surface sterilization. To sterilize the roots and stems samples were treated with 70% ethanol for 50 seconds and 1% sodium hypochlorite for 15 minutes. They were then rinsed with double distilled water for 5 minutes. To verify the efficacy of the sterilization process, water is collected after final wash and inoculated onto triplicate plates containing nutrient agar media (Himedia, India). Once surface contamination-free samples were obtained, they were crushed with the help of mortar and pistil and samples are spread over LB agar [36]. Following aerobic incubation at 30°C for a period of 3 days, individual colonies were chosen and assessed for their nitrogen-fixing capability as endophytes of Kaladani rice. Colonies that developed on NA media were transferred to the nitrogen-free $JNFb^{-}$ solid agar medium. The colonies exhibiting distinct morphological characteristics on $JNFb^{-}$ agar medium was selected and sub-cultured. To ensure the diazotrophic nature of the isolates, individual isolates were sub-cultured 4 times on JNFb- agar plates.

**Molecular characterization of Endophytic Bacteria.**

For molecular identification of the endophytic bacterial isolates, the 16S rRNA gene was amplified and sequenced. Genomic DNA was extracted from the bacterial colonies for this purpose. A bacterial colony was picked up and suspended in 100 µl of ddH2O. To extract genomic DNA, the bacterial sample was lysed by boiling it for 10 minutes and then freezing it for 5 minutes. The lysed sample was subsequently centrifuged at 13,000 rpm, and the resulting supernatant was utilized as the template for PCR. The amplification of the 16S rRNA gene was carried out using universal primers 27F and 1492R [37]. In the 25 µl PCR reaction mixer, 1 µl (10.0 ng) of template DNA was combined with 12.5 µl of (2X) PCR master mix (Promega Group), 0.5 µl (10 µM) of 27F primers and 0.5 µl (10 µM) 1492R primer were taken. 10.5 µl of ddH2O. The PCR conditions were programmed as follows: Initial denaturation at 98°C for 5 minutes, denaturation at 94°C for 45 seconds, annealing at 58°C for 1 minute, amplification at 72°C for 2 minutes, and a extension at 72°C for 10 minutes. Following the PCR amplification, the resulting PCR product was purified using the Takara quick PCR Purification Kit (Takara). The purified product was then subjected to sequencing using the ABI 3730 sequencer, following the protocols provided by the manufacturer, through Nano Scientific Pvt. Ltd. in Hyderabad. For the phylogenetic analysis of endophytic bacteria. The obtained 16S rRNA gene sequences were compared to the existing homologous sequences in the NCBI database using the BLAST (Basic Local Alignment Search Tool) algorithm [38].

| Endophytes from Kaladani Roots (KDR) | | Endophytes from Kaladani Stem (KDS) | |
|---|---|---|---|
| KDR 1 | + | KDS 2 | _ |
| KDR 3 | + | KDS 4 | + |
| KDR 4 | + | KDS 5 | + |
| KDR 7 | + | KDS 6 | + |
| KDR 8 | _ | KDS 9 | + |
| KDR 9 | + | KDS 10 | + |
| KDR 10 | _ | KDS 11 | + |




**Phosphate utilization**

To assess the phosphate solubilization capability of the endophytic bacterial strain, a qualitative analysis was conducted following the method described by Mehta and Nautiyal [39]. The endophytic bacterial strain was cultured in NBRIP medium (0.02% KCl, 0.05% $(NH_4)_2SO_4$, 0.02% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$, 1% glucose, 0.05% yeast extract, 0.0002% $FeSO_4 \cdot 7H_2O$, 0.0002% $MnSO_4 \cdot H_2O$, and 2% agar). The strain was grown on NBRIP medium containing tricalcium phosphate ($Ca_3(PO_4)_2$) as the exclusive source of phosphate. After 8 days of incubation, clear zones around the colonies were observed, indicating the solubilization of inorganic phosphate.

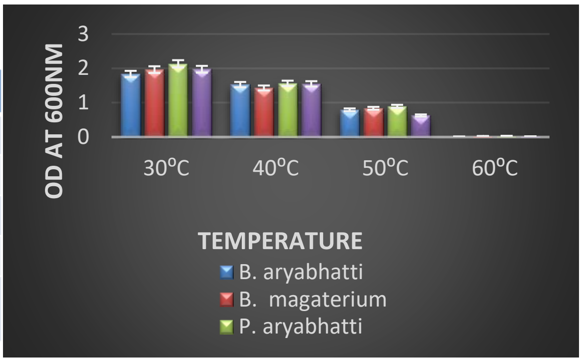
**Abiotic stress: Challenging climate change**

Abiotic stresses often arise from shifts in climate. The impact of abiotic stress on plant development and yield is evident amid the evolving ecological consequences of climate change (Bellard et al., 2012). This poses a significant threat to crop production, especially with the recent surge in agricultural output driven by a rapidly growing human population vying for environmental resources (Wallace et al., 2003). Agriculture, notably rice production, is particularly susceptible to the effects of climate change (Rosenzweig et al., 2014). Abiotic plant stress, encompassing various environmental factors such as UV radiation, drought, salinity, high and low temperatures, etc., could ultimately result from severe climatic changes, posing risks to rice crops. The plant may also face multiple stresses simultaneously due to severe climate changes, such as a combination of drought and high-temperature stress, leading to a unique and unpredictable stress condition that cannot be anticipated from individual stressors (Suzuki et al., 2014). While plants have the ability to adapt to changing climatic conditions (Yoshida et al., 2014), the simultaneous impact of multiple stressors resulting from the frequently changing climate can lead to the complete destruction of a crop. An environment that may be suitable for one plant genotype can impose various abiotic stresses on a different genotype with a distinct adaptive response, highlighting the complexity of the situation (Des Marais et al., 2013).

**Temperature tolerance**

Plants have optimal temperature ranges for growth and development. High temperatures can cause heat stress, which can damage or kill plants. Low temperatures can cause chilling injury or frost damage. Isolated strains are grown in nutrient broth medium with different temperature 30ºC 40ºC 50ºC and 60ºC for 48 hours then OD taken at 530 nm to see the maximum growth of endophytes at different temperature.

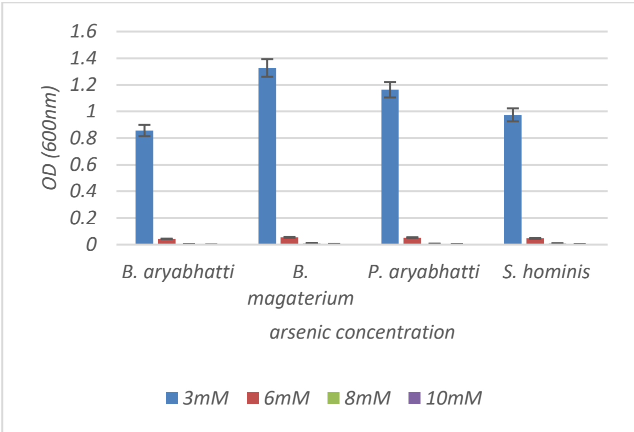
| Temperature tolerant (OD at 600nm after 48 hours) | | | | |
|---|---|---|---|---|
| Strain Name | 30°C | 40°C | 50°C | 60°C |
| *Bacillus aryabhatti* | 1.836 | 1.527 | 0.794 | 0.012 |
| *Bacillus magaterium* | 1.963 | 1.429 | 0.837 | 0.026 |
| *Preistia aryabhatti* | 2.132 | 1.563 | 0.894 | 0.031 |
| *Staphyllococcus hominis* | 1.974 | 1.549 | 0.627 | 0.019 |




## Arsenic tolerance

Arsenic is a toxic element that can accumulate in soils and water sources. Plants can absorb arsenic through their roots, and high levels of arsenic can cause plant stunting, yellowing of leaves, and reduced yields. Strains grown in nutrient broth medium contain 3mM, 6mM and 8mM concentration of sodium arsenite. Incubate the culture at 30°C for 72 hours. Taken OD absorption at 530 nm to see the growth of endophytes and estimate the MIC.

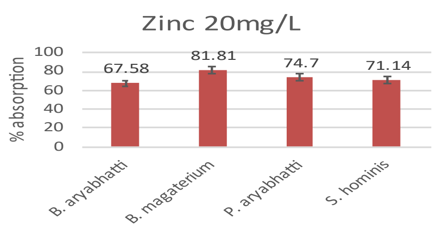
| Sodium arsenite (OD 600nm) | | | | |
|---|---|---|---|---|
| Strain Name | 3mM | 6mM | 8mM | 10mM |
| *Bacillus aryabhatti* | 0.857± 0.0314 | 0.0428± 0.0022 | 0.002 | 0 |
| *Bacillus magaterium* | 1.327± 0.0431 | 0.0547± 0.0019 | 0.019 | 0.004 |
| *Preistia aryabhatti* | 1.163± 0.00251 | 0.0418± 0.0024 | 0.005 | 0.002 |
| *S. hominis* | 0.974± 0.00273 | 0.458± 0.0224 | 0.018 | 0.001 |



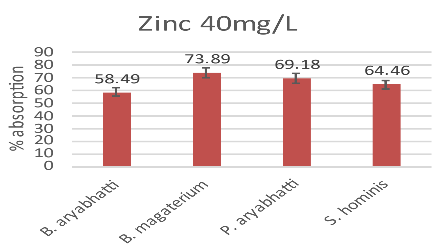

## Zinc absorption

Zinc is an essential micronutrient for plants, but high levels of zinc can be toxic to plants, causing leaf chlorosis and reduced yields. Zinc toxicity is more likely to occur in acidic soils with a low pH. Strains are grown in nutrient broth medium contains 20mg/L and 40mg/L zinc sulphate concentration and incubate at 30°C for 72 hours. Taken absorption at 220 nm to see the growth of endophytes in culture medium to estimate zinc utilization.

| Zinc Sulphate 20mg/L (OD 220nm) | | | |
|---|---|---|---|
| Strain Name | Control | OD (220 nm) After 72hours | % absorption of Zinc |
| *Bacillus aryabhatti* | 0.253 | 0.082 | 67.58 |
| *Bacillus magaterium* | 0.253 | 0.046 | 81.81 |
| *Preistia aryabhatti* | 0.253 | 0.064 | 74.70 |
| *Staphyllococcus hominis* | 0.253 | 0.073 | 71.14 |



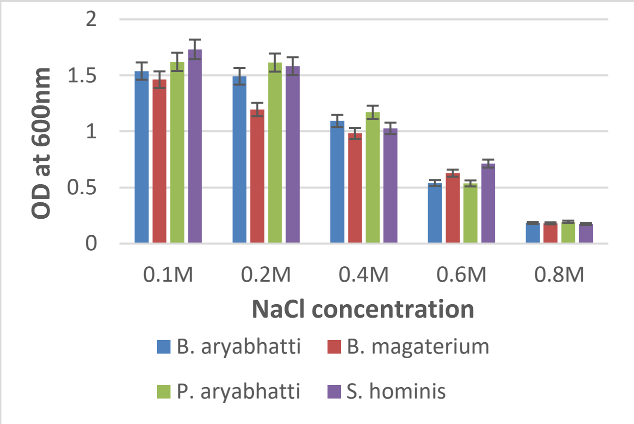

| Zinc Sulphate 40mg/L (220nm) | | | |
|---|---|---|---|
| Strain Name | Control | OD (220 nm) After 72hours | % absorption of Zinc |
| *Bacillus aryabhatti* | 0.318 | 0.132 | 58.49 |
| *Bacillus magaterium* | 0.318 | 0.083 | 73.89 |
| *Preistia aryabhatti* | 0.318 | 0.098 | 69.18 |
| *Staphyllococcus hominis* | 0.318 | 0.113 | 64.46 |



**Salt stress**

High levels of salt in soil or water can be toxic to plants, causing reduced growth and yield. Salt stress can also cause water stress, as high salt levels can draw water away from the plant roots. Some plants, such as halophytes, have adapted to grow in high-salt environments. Isolated strains are grown in nutrient broth medium with 0.1M, 0.2M, 0.4m, 0.6M and 0.8M NaCl concentration. Incubate culture at 30°C for 48 hours. Absorption is taken at 530 nm to see the optimum growth of endophytes with different salt concentration.

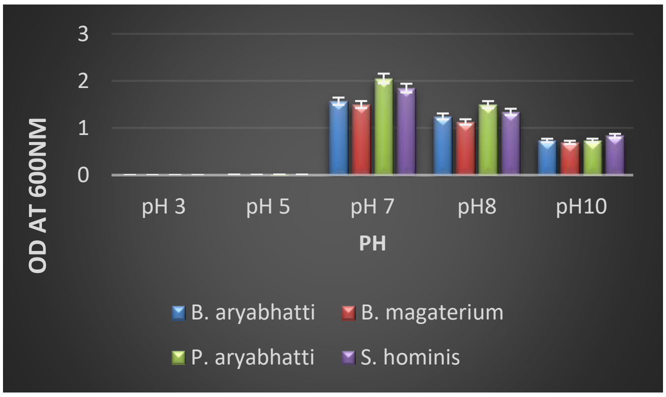
| Salt (NaCl) stress (OD at 600nm after 48 hrs) | | | | | |
|---|---|---|---|---|---|
| Strain Name | 0.1M | 0.2M | 0.4M | 0.6M | 0.8M |
| *Bacillus aryabhatti* | 1.538 | 1.492 | 1.094 | 0.538 | 0.185 |
| *Bacillus magaterium* | 1.462 | 1.196 | 0.983 | 0.628 | 0.180 |
| *Preistia aryabhatti* | 1.621 | 1.614 | 1.171 | 0.536 | 0.195 |
| *Staphylloccus hominis* | 1.732 | 1.583 | 1.027 | 0.713 | 0.176 |




**pH tolerance**

Soil pH is an important factor for plant growth and can affect nutrient availability. Most plants prefer a slightly acidic to neutral soil pH between 6.0 and 7.5. Strains grown in nutrient broth medium with different pH (3,5,7,8,10). Incubated the culture at 30°C for 48 hours then OD taken at 530 nm to see the maximum growth of endophytes at different PH.

| pH stress ( OD at 600nm after 48 hrs) | | | | | |
|---|---|---|---|---|---|
| Strain Name | pH 3 | pH 5 | pH 7 | pH8 | pH10 |
| *Bacillus aryabhatti* | 0 | 0.008 | 1.568 | 1.247 | 0.735 |
| *Bacillus magaterium* | 0 | 0.005 | 1.498 | 1.129 | 0.694 |
| *Preistia aryabhatti* | 0 | 0.012 | 2.052 | 1.496 | 0.736 |
| *Staphylloccus hominis* | 0 | 0.007 | 1.848 | 1.342 | 0.834 |




**Result**

Diazotrophic endophytic bacteria were isolated from stems and roots of Kaladani rice (Table1). The number of diazotrophic endophytic bacterial colonies were in the range of $10^3$ to $10^4$ per gram (fresh weight) of the stems and roots. Fourteen of the tested isolates yielded a predominant morphotype following additional purification of the diazotrophic isolates. Sequencing of PCR product analysis showed that four different Diazotrophic endophytes *Bacillus aryabhattai* (accession no. OQ426536). *Priestia aryabhatti* (accession no. OQ422565). *Bacillus magaterium* (accession no. OQ422490). *Staphycoccus hominis* (accession no. OQ422489). four of these isolates, chosen for Phosphate utilization and abiotic stress tolerant test. All these isolates are capable to utilize phosphate. All the 4 identified endophytic strains tested positive for phosphate utilization. They have shown tolerance to high concentrations of zinc 40mg/L. *Bacillus magaterium* strain shown the maximum uptake of zinc. *Bacillus magaterium* shown highest MIC (10mM) for sodium Arsenite. All the 4 identified strains grown on alkaline pH up to pH10 but *Staphylococcus hominis* is more tolerant to alkaline pH. *Preistia aryabhatti* shown growth at 50°C little more tolerance at high temperature as compared to other.

## Discussion

Various diazotrophic bacteria were obtained from the roots and stems of Kaladani rice variety Diverse diazotrophic bacteria were isolated from the surface-sterilized roots and stems of Kaladani rice variety growing on nutrient agar medium. Twenty-one diazotrophic bacteria (Table 1). The population of heterotrophic bacteria that could be cultured from the surface-sterilized roots and stems of Kaladani rice variety accounted for only 5 to 10% of the total bacteria present, which aligns with the previous observations made by Barraquio et al. (40). Among these, a specific group of N2-fixing bacteria was isolated from the surface-sterilized stems and roots. were identified as Bacillus aryabhattai, Priestia aryabhatti, Bacillus magaterium and Staphycoccus hominis based on 16S rDNA sequence. Although Staphylococcus hominis subsp. group of N2-fixing bacteria exhibited notable abilities as plant growth promoters and demonstrated an antagonistic effect against fungal leaf spot disease in groundnuts. The disease was caused by a distinct strain of Pithomyces atro-olivaceous. These bacteria showed a preference for medicinal plants, which served as their favorable ecological niche. (41). Extensive in-vitro testing revealed Priestia aryabhattai to be a highly effective crop growth promoter capable of withstanding multiple stresses. It exhibited various plant growth-promoting characteristics, including efficient solubilization of nutrients such as potassium(K), zinc (Zn) and phosphate(P) and synthesis of some extracellular enzymes like amylase, cellulose, protease, pectinase and lipase and These bacteria also displayed antagonistic activity against prominent fungal pathogens such as Rhizoctonia solani, Alternaria solani, Ustilaginoidea viren and Fusarium oxysporum,. Additionally, Priestia aryabhattai exhibited increased levels of siderophores, indole-3-acetic acid, K, Zn and Phosphate solubilization, ammonia synthesis and ACC under elevated salt conditions (42). The growth-promoting effects of Bacillus aryabhattai on plants were observed, along with the associated transcriptional changes. This endophytic bacterium positively influenced the growth of tobacco and Arabidopsis plants. By studying Arabidopsis plants treated with this bacterium, researchers identified a set of highly expressed genes. These included apyrase, cinnamyl alcohol dehydrogenase, benzaldehyde dehydrogenase, indole acetaldoxime dehydratase, thioredoxin H8 and gibberellin-regulated protein (43). The growth-promoting properties of a Bacillus megaterium strain were observed in both A. thaliana (Arabidopsis thaliana) and P. vulgaris (common bean) seedlings. To gain more insights into the involvement of cytokinin signaling in plant responses to bacterial inoculation, researchers conducted additional investigations using mutants of A. thaliana lacking certain putative cytokinin receptors. AHK2, AHK3 andCRE1, as well as the RPN12 These genes, AHK2 and RPN12, are known to be involved in cytokinin signalling pathways. The study showed that the plant growth promotion effects of B. megaterium were diminished in single and double mutant combinations of AHK2 and also in the RPN12 mutant. (44).

## Conclusion

The screening of endophytic bacteria capable of nitrogen fixing and the utilization of phosphate plays a crucial role in enhancing the overall growth of rice plants. In this study, we have isolated four different endophytic bacterial strains., *Bacillus aryabhattai* (OQ426536). *Priestia aryabhatti* (OQ422565). *Bacillus magaterium* (OQ422490).*Staphycoccus hominis* (OQ422489) from the rice roots which not only nitrogen fixing bacteria in addition also simultaneously had phosphate solubilization activity. Since the current scenario is facing the urge for food to satisfy the hunger of the increasing human population, developing bioinoculant agents with these endophytes may increase the productivity of rice crops. the potential for utilizing endophytic bacteria, which promote plant growth, and the prospects of developing endophytic bacterial formulations as a viable alternative to chemical inputs in the near future for sustainable agriculture.

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## Conflict of interest

The author state that they have no conflict of interest related to the publication of this paper.

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