Halotolerant Phosphat Solubilizing Bacteria from Paddy Saline Soil

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Abstract. Salinity stress on productive rice fields in coastal areas will have a negative impact on productivity through osmotic stress and nutrient deficiencies. Salinity stress causes high levels of Na and Cl in the soil, thus inhibiting phosphate absorption. Utilization of indigenous P-solubilizing bacteria from saline land is an alternative, environmentally friendly technology. The purpose of this study was to isolate and characterize P-solubilizing bacteria from saline rice fields. Soil samples were taken from the rice rhizosphere in Nyamplungsari Village, Peraturkan District, Pemalang Regency. Isolation and characterization were carried out at the Agronomy & Horticulture Laboratory, Faculty of Agriculture, UNSOED. The variables observed included P solubility index, P solubilizing ability, IAA production, and bacterial identification using the 16S RNA method. The results of the study obtained 7 isolates of P-solubilizing bacteria that had the ability to solubilize P and produce IAA. Isolate KF is a P-solubilizing bacterium that has the highest P-solubilizing ability and is identified as *Priestia megaterium* strain NRRL B-350. *Priestia megaterium* strain NRRL B-350 is a species of P-solubilizing bacteria that has the potential to be developed as a specific biological fertilizer for saline soil to increase the growth and yield of rice plants under saline stress.

Key words: rice; bacteria; solubilizing; P; IAA.

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INTRODUCTION

Salinity is a global problem that continues to increase along with increasing global warming. In Indonesia, salinity generally occurs in coastal areas or areas affected by seawater intrusion. Seawater intrusion is caused by rising sea levels combined with lowering land surfaces, low discharge, and groundwater extraction, causing increased salinity (Tarolli et al., 2024). Increased salinity hurts agricultural productivity as a complex impact, both in terms of morphology, physiology, biochemical processes, such as seed germination, plant growth, and water and nutrient absorption (Shrivastava & Kumar, 2015). According to El-ramady et al. (2024). Plant damage due to salinity stress is mainly caused by ionic, osmotic, and oxidative stress. Salinity has

an impact on microbial activity and the diversity of soil microbes that play a role in vital activities in the soil, such as residue decomposition, soil respiration, and nitrogen transformation, and in general, in saline conditions, plants will be deficient in the nutrients N, Ca, K, P, Fe, and Zn (Mishra *et al.*, 2023; Shrivastava and Kumar, 2015).

Phosphate is one of the essential macronutrients needed by plants, and in saline conditions, its solubility and availability are low. Saline conditions cause high levels of Na⁺ and Cl⁻ ions, causing toxicity in plants and inhibiting the absorption of nutrients such as Ca²⁺, K⁺, and P (Xie et al., 2022). Su et al., (2022) reported that increasing salinity hurts plant P uptake and causes P deficiency. Shahriaripour et al., (2015) also reported that increasing salinity had an impact on

decreasing leaf growth, stems, root dry weight, and P uptake by pistachio seedling roots.

One strategy in managing plants in saline environments can be done by utilizing beneficial microbes that can dissolve P and reduce the impact of salinity toxicity on plants. Beneficial microbes colonize the rhizosphere of plants and stimulate growth through both direct and mechanisms (Shrivastava & Kumar, 2015). The use of phosphate-solubilizing bacteria in plant cultivation under saline stress conditions is one strategy to increase phosphate availability and support plant growth (Hoesain et al., 2024). Psolubilizing bacteria can increase P availability through the mechanism of solubilization and mineralization processes by transforming unavailable P into bioavailable P in the soil. The mobilization mechanism of P nutrients based on the form of unavailable P is for inorganic P, the solubilization mechanism is through the process of acidification, protonation, chelation, and EPS production; while for organic P, the mineralization process is through enzymatic processes such as phosphatase, phytase, phosphonatases, and C-P lyases (Dey et al., 2021).

The effectiveness of the use of phosphatesolubilizing bacteria is highly dependent on the adaptation and origin of the bacteria. The use of indigenous rhizobacteria is one strategy to increase the effectiveness of P solubilization because of their high level of adaptation, so that the ability to colonize the roots will be high. The North Coast of Pemalang is one of the centers of rice production; however, in some locations, it is very close to the beach, so that intrusion occurs and causes an increase in salt levels in irrigation water, and interferes with plant growth. Isolation and characterization of P-solubilizing bacteria from the area are very important to obtain superior P-solubilizing bacterial candidates to stimulate rice plant growth.

Various previous studies have reported on the diversity and ability of P-solubilizing bacteria in increasing P availability and plant growth. Tat, (2024) reported that Burkholderia vietnamiensis 1.7 and Priestia aryabhattai 6.1 are bacterial species that are classified as capable of dissolving P in liquid NBRIP enriched with NaCl at various concentrations between 1.0 – 5.0 percent. Furthermore, Jiang et al., (2021) obtained three bacterial phyla, Acidobacteria, Chloroflexi, and Planctomycetes, from the peanut plant rhizosphere, and were able to increase P availability in high-salt soil conditions, and were able to increase peanut plant growth. Setiawati et al., (2022) reported that *Bacillus valezensis*, *Bacillus* sp, and *Bacillus subtilis* had P solubilization ability with a P solubility index ranging from 1.06 to 2.52 in media with a salt content of up to 3 percent.

Indigenous P-solubilizing bacteria in saline paddy soils play a significant role in increasing P availability for rice plant growth. Indigenous Psolubilizing bacteria will be more effective because they will be more adaptive, so that their population will grow quickly, so that the amount of P dissolved will be greater, and able to help plants grow and absorb P (Sen et al., 2024). The discovery of indigenous isolates in saline rice paddy soils opens up opportunities for developing effective biofertilizers in the future to increase P availability, P fertilization efficiency, and reduce the use of inorganic P fertilizers, as well as improve rice plant growth and yield in saline soils. This study aimed to isolate, characterize, and obtain indigenous P-solubilizing bacteria from saline paddy soil on the North Coast of Pemalang.

METHODS

Research sites

This research was conducted at The Agronomy & Horticulture Laboratory, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, from October 2021 to June 2022. Soil samples were taken from the rice plant rhizosphere in rice fields in Nyamplungsari Village, Petarukan District, Pemalang Regency, Central Java.

Isolation and Phosphat Solubilizing Assay

The soil sample was weighed as much as 5 g, put into a 500 ml Erlenmeyer flask containing 500 ml of physiological solution, and then a dilution series was carried out up to 10⁻⁸. As much as 0.1 ml of solution at a dilution of 10⁻⁸ was inoculated in a petri dish containing Pikovskaya media, then incubated for 24 hours. Bacteria that can dissolve P will be characterized by the formation of a clear zone (halo zone) on the edge of the colony. The diameter of the clear zone and the diameter of the bacterial colony were measured, and then the P Solubility Index (IP) value was calculated using the following formula:

$$IP = \frac{diameter\ of\ halo\ zone - diameter\ of\ colony}{diameter\ of\ colony}$$

Testing the ability of P-solubilizing bacteria to dissolve P was carried out using the spectrophotometric method (Thant et al., 2018). A

total of 1 ose of bacterial isolate was inoculated into 250 mL of liquid Pikovskaya media and incubated for 15 days at room temperature and shaken at a speed of 120 rpm. After 15 days of incubation, the solution was filtered with Whatman filter paper number 1 to obtain 20 ml of solution. A total of 13 ml was taken and centrifuged for 15 minutes at a speed of 1000 rpm. A total of 5 ml of supernatant was taken, and 1 ml of KH₂PO₄ (0.295 g/100 ml) was added to make a stock solution of 1000 ppm, and diluted to make solutions with concentrations of 0 ppm, 2 ppm, 4 ppm, 6 ppm, 8 ppm, and 10 ppm. The sample and stock solution that had been added with concentrated P reagent were incubated for 30 minutes. P dissolution was measured with a spectrophotometer at a wavelength of 693 nm.

IAA Assay

The ability of P-solubilizing bacteria to IAA was measured by produce spectrophotometric method (Rini et al., 2020). A total of 1 ose of bacterial isolate was inoculated into 50 mL of Nutrient Broth (NB) media supplemented with 0.1 percent L-tryptophan, then incubated for 24 hours at room temperature and shaken at 120 rpm. 1 ml of inoculum was taken and put into 100 ml of NB + L-tryptophan 0.1% media, then incubated for 6 days at room temperature with a shaker (120 rpm). 0.5 ml of supernatant was taken and put into a test tube, and 1.5 ml of Salkowski reagent was added (250 mL of distilled water, 150 mL of H2SO4, and FeCl₃.6H₂O, namely 7.5 mL) and incubated in a dark room. The formation of IAA will be marked by a color change from orange to red or purple. The absorbance value was measured using a spectrophotometer with a wavelength of 530 nm. The IAA content was calculated using a standard curve of pure IAA.

Molecular Identification of Selected Isolate

DNA extraction procedures followed the protocol of the Quick-DNA Magbead Plus Kit (Zymo Research, D4082). The molecular identification of bacterial isolates was conducted based on 16S rRNA universal primers (27F and 1492R). Amplification of 16S rRNA was conducted using My Taq HS Red Mix, 2X (Bioline, BIO-25048). The PCR products were sequenced based on Samger DNA Sequencing by Capillary Electrophoresis. Assembly of contigs was conducted using BioEdit. The sequences were analyzed using BLAST to identify closely related species. Phylogenetic analysis was

performed using the neighbor-joining method in MEGA software ver 11 with bootstrap analysis (1,000 replicates) to confirm evolutionary relationships.

RESULTS AND DISCUSSION

P-Solubilizing Capacity

The study results have obtained as many as 7 isolates of P-solubilizing bacteria isolated from the rhizosphere of plants in rice fields experiencing salt stress. All isolates that were successfully isolated showed the ability to dissolve P. This can be seen from the fact that each isolate formed a clear zone in culture with Pykovkaya media. The ability of microbes to form a clear zone is closely related to the amount of P that can be dissolved qualitatively (Sembiring and Sabrina, 2022; Prameselly et al., 2024). The ability of P-solubilizing bacteria to dissolve P through various mechanisms, including both enzymatic mechanisms and organic acid production mechanisms. The formation of a clear zone around the colony indicates the ability to dissolve P, which occurs due to the decreasing pH of the media as an increase in phosphatase enzyme activity. Bacteria produce organic acids that cause the pH value to decrease by interacting with P bound to various ions such as Al³⁺, Fe³⁺, Ca²⁺, and Mg²⁺, so that it becomes an organic form and dissolves as free P ions (Tarigan et al., 2023).

Table 1. P Solubilizing Ability of Bacterial Isolates from the Rhizosphere of rice plants on the North Coast of Java Saline Soil

No	Isolate	Phosphat Solubilizing Index	Available Phosphat (ppm)
1	KF	2.10	4.77
2	KF1	2.22	3.63
3	IPF1	2.32	3.38
4	JF4	2.23	4.60
5	E6	2.19	3.45
6	E16	2.39	3.78
7	E17	2.09	1.61

The potential ability of bacterial isolates to qualitatively dissolve can be estimated through the P solubility index value. The results of the study showed that the P solubility index value in various isolates ranged from 2.10 to 2.39. The highest P solubility index value was achieved in isolate E16 at 2.39, followed by isolates IPF1, JF4, KF1, E6, KF, and E17 with P solubility index values of 2.32, 2.23, 2.22, 2.10, and 2.09, respectively (Table 1). The variation in the P solubility index

value of bacteria indicates the diversity of their ability to dissolve P. The larger the P solubility index value, indicates the ability of the bacteria to dissolve P (Tarigan et al., 2023).

Screening of Rhizobacterium's ability to dissolve phosphate is not accurate enough based only on the formation of a clear zone or the P solubility index value. According to Lynn et al., (2014) that the formation of a clear zone is only based on cell growth, production of organic acids, and H+ ions from undissolved P. Therefore, the determination of P dissolving ability is carried out quantitatively. The results of the study showed that out of 7 isolates of P-solubilizing bacteria, they were able to dissolve P quantitatively with values ranging from 1.61 to 4.77 ppm (Table 1). These results indicate that the KF isolate is the bacterial isolate with the highest solubilizing ability of 4.77 ppm. These results are in line with previous studies. Adibe et al., (2022) reported that the genus Bacillus spp. was able to dissolve P of 0.626 mg P/l in 10 days of incubation. Rhizobacteria can increase the availability of P in the soil by dissolving P in inorganic phosphate and organic phosphate (Marpaung et al., 2024). According to Rajguru & Bhatt, (2022), the mechanism of P dissolution by rhizobacteria is through the production of organic acids such as gluconic acid, oxalic acid, succinic acid, citric acid, and lactic acid, resulting in acidification of microbial cells, and as a result, P will be released from phosphate minerals through proton substitution.

IAA Production

The results showed that all isolates of Psolubilizing bacteria were able to produce IAA with values ranging from 2.69 to 6.00 ppm. The highest IAA production capacity was achieved in isolate E16 with an IAA production capacity of 6.00 ppm, and the lowest IAA value of 2.69 was produced by isolate KF (Table 2). Isolate E16 showed the highest IAA production capacity, reaching 80.54 percent higher than the average IAA production of other isolates. The isolates of P-solubilizing bacteria that had been isolated had an average IAA production capacity of 3.23 ppm. IAA is a growth stimulant that acts as a growth promoter, especially in the cell division process in the meristematic tissue, so that the ability to produce IAA will have a positive impact on plant growth by improving plant root growth.

The ability of all P-solubilizing bacterial isolates to produce IAA can be classified as Plant Growth Promoting Rhizobacteria (PGPR). This is

in accordance with Rini et al., (2020) that the characteristics of rhizobacteria as PGPR are characterized by the ability to produce IAA as a growth substance. The results of this study indicate that the ability of P-solubilizing bacterial isolates to produce IAA varies among isolates. IAA is a phytohormone that is crucial for plant growth, particularly in stimulating cell division in meristematic regions. PGPR's ability to produce IAA varies widely between species and strains, influenced by culture conditions, particularly the availability of precursors in the form of L-tryptophan, which naturally originates from root exudates (Lebrazi et al., 2020). Joshi et al., (2021) stated that to increase IAA production with the addition of L-tryptophan, and without Ltryptophan, it will decrease, so this shows that the ability to produce IAA depends on the presence of L-tryptophan as a precursor to IAA biosynthesis.

The diversity of P-solubilizing bacteria's ability to produce IAA is in line with the results of previous researchers. Kadmiri et al., (2018) reported that Azotobacter brasilense DSM1690 was able to produce IAA in saline conditions in the range of 22 µg ml⁻¹ IAA. Furthermore, Mohan et al., (2020) also reported that Bacillus cereus is a P-solubilizing bacterium that is tolerant to saline environmental conditions and has the ability to produce IAA. Kouas et al., (2024) also reported that Bacillus sp., Bacillus cereus, Pseudomonas brassicacearum, and Pseudomonas solubilizing bacteria that are also able to produce high IAA, and application to barley plants can stimulate root growth and increase P nutrient uptake in deficient conditions.

Table 2. The Ability to Produce IAA from Bacterial Isolates Originating from the Rhizosphere of Rice Plants on The North Coast of Java.

No	Isolate	IAA Production (ppm)
1	KF	2.69
2	KF1	3.84
3	IPF1	2.94
4	JF4	3.74
5	E6	3.48
6	E16	6.00
7	E17	3.25

Identification of selected bacteria

The Molecular identification was carried out on isolates that had the highest P solubilization potential. Based on the results of the study, isolate KF was the isolate that was able to dissolve the highest P compared to other isolates, which was 4.77 ppm. Based on the results of molecular analysis using the 16S RNA gene method, it produced a DNA band of around 1422 bp was produced (Figure 3). The results of molecular sequencing analysis using 16S RNA showed that isolate KF was a bacterium with the genus *Priestia* sp. Based on the phylogenetic tree analysis, it showed that isolate KF was in the same cluster as *Priestia megaterium* strains NRRL B-350 (Figure 4).

Based on the results of the analysis, it was shown that the KF isolate was identified as *Priestia megaterium* strain NRRL B-350. This bacterium is a mesophilic bacterium belonging to the Bacillaceae family. According to Gupta *et al.*, (2020) *Priestia megaterium* has another name as *Bacillus megaterium*. The results of the study showed that *Priestia megaterium* strains NRRL B-350 can act as growth-stimulating bacteria as well as P-solubilizing bacteria that are resistant to saline environments. This is reinforced by the results of previous studies. Kang *et al.*, (2014) reported that *B. megaterium* mj1212 has characteristics as a growth promoter and P-solubilizing activity, where *B. megaterium*

mj1212 cultured in a medium containing phosphate showed a halo zone that increased significantly. Thant *et al.*, (2018) reported that *B. megaterium* is tolerant to salt stress up to a concentration of 6 percent, and the solubility of P in solution increases with increasing incubation time.

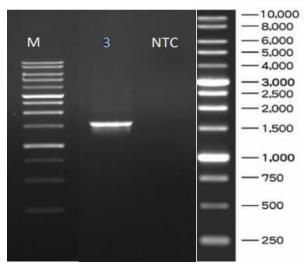


Fig 3. Electrophoresis-Product Amplification of the 16S rRNA gene

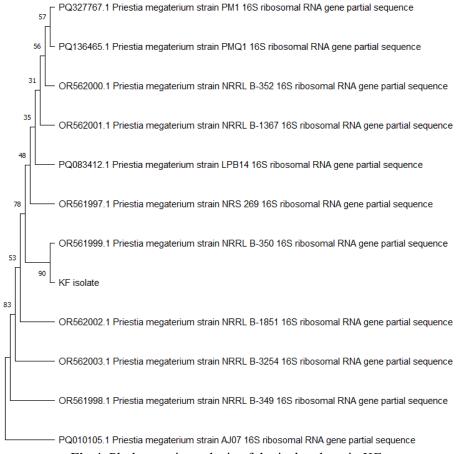


Fig 4. Phylogenetic analysis of the isolated strain KF

Priestia megaterium strain NRRL B-350 is a new species originating from the rhizosphere of rice plants in saline soil environments in Indonesia. Priestia megaterium strain NRRL B-350, found in this study, can produce IAA in addition to the ability to solubilize phosphate, making it very promising as a plant growthpromoting rhizobacterium. As reported by Zhu et al., (2023), Bacillus megaterium OQ560352 can increase the growth of corn plants with a combination of NPK applications, where there is an increase in shoot growth, absorption, and increased P availability in the soil. Furthermore, de Oliveira-Paiva et al., (2024) reported that Bacillus megaterium B119 combined with B. subtilis B2084 is effective in increasing corn yields with an increase in yield between 11-24 percent. These results open up opportunities in the development of saline-tolerant P-solubilizing bacterial bio-inoculants to increase the growth and yield of rice plants in saline soils, both in the form of single inoculants and in the form of microbial consortia.

CONCLUSION

Based on the research results, it can be concluded that 7 isolates of rhizobacteria were obtained from saline-stressed rice fields with the ability to solubilize bacteria and can produce IAA. Isolate KF is a P-solubilizing bacterium that has the highest P-solubilizing ability and is identified as Priestia megaterium strain NRRL B-350. Priestia megaterium strain NRRL B-350 is a species of P-solubilizing bacteria that has the potential to be developed as a specific biological fertilizer for saline soil to increase the growth and vield of rice plants under saline stress. The results of this study open up opportunities for further testing regarding the suitability and synergy of Priestia megaterium strain NRRL B-350 with other rhizobacteria species, so that it can provide effective bio-inoculants with various capabilities in supporting the growth and yield of rice plants on saline land.

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AUTHOR CONTRIBUTION STATEMENT

All authors contributed significantly to the reported manuscript. work in this Conceptualization, sampling, analysis, data & cuarion. Writing review editing. Investigation, Methodology; RS: laboratory analysis, sampling and writing, DALA: laboratory analysis, isolation, characterization, and writing; NWAL: Conceptualization, sampling, analysis, writing; REKK: data analysis, Writing – review & Conceptualization. editing: EO: molecular analysis, data curation, Writing; OAMO: Writing – review & editing.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that no artificial intelligence (AI) tools were used in the generation, analysis, or writing of this manuscript. All aspects of the research, including data collection, interpretation, and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies.

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