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Screening and bioassay of halotolerant phosphate solubilizing bacteria using rice (*Oryza sativa* L.) on salinity media

Betty Natalie Fitriatin[1*], Diya Septiani Lukita[2], Debora Dellaocto Melati Ambarita[2], Mieke Rochimi Setiawati[1] and Tualar Simarmata[1]

[1] *University of Padjadjaran – Raya, Department of Soil Science and Land Resources, Faculty of Agriculture, Jatinangor 45363, Indonesia*
[2] *University of Padjadjaran – Raya, Agrotechnology, Faculty of Agriculture, Jatinangor 45363, Indonesia*
[*]*Corresponding author:* betty.natalie@unpad.ac.id

Abstract

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Some beneficial microbes have the ability to tolerate salinity stress conditions to obtain superior isolates of halo tolerant phosphate solubilizing bacteria (PSB) and determine the ability of PSB isolates to increase the growth of rice seedlings. The research was carried out at the Laboratory and Greenhouse of the Department of Soil Science and Land Resources, Faculty of Agriculture, Universitas Padjadjaran. In this study, halotolerant PSB were isolated from saline soil with salinity 4 dS/m and grown in Pikovskaya medium. The results showed that a superior isolate of phosphate solubilizing bacteria had been obtained from saline soil which were capable of producing phytohormones, phosphatase enzymes and organic acids. Three superior isolates PSB 1, PSB 3, and PSB 6 produced phosphatase enzymes (3.508 µg pNP/g/h, 3.307 µg pNP/g/h, and 3.257 µg pNP/g/h), growth hormone IAA (4.611 ppm, 4.913 ppm, and 3.819 ppm), and organic acids (citric, acetic, oxalic, lactic, malic and ascorbic). These isolates could form biofilms . Inoculation of PSB isolates were able to increase root length by 45.01%, 37.54%, and 32.65%. The isolate was also able to increase plant height by 28.58%, 43.77%, and 62.69%.The provision of selected PSB isolates, could increase the growth of rice plants and have the potential to be developed as biofertlizer to increase the productivity of rice plants in saline soil.

Keywords: IAA Phytohormones; phosphatase; organic acid; salinity; rice plants

Introduction

The Rice plant (*Oryza sativa* L.) is the largest food source because in Indonesia rice is a staple food. The need for basic food is increasing along with the increase in population. According to statistic data at 2022 the rice consumption needs of the population in Indonesia reached 30.03 million tons. The condition of the rice fields is increasingly disappearing due to the conversion of land functions so its location is increasingly displaced to the coastal area.

However, most of the rice fields on the coast experience salinity caused by climate change (Arifah et al., 2022). Saline soil has a high content of soluble salts (NaCl, Na_2CO_3, Na_2SO_4), which affects plant growth (Irakoze et al., 2021). Salinity is an inhibiting factor in the growth and development of rice plants in saline areas. Efforts to increase nutrient availability, tolerance, and plant productivity can be done by using halo tolerant biofertilizer. Biofertilizers are inoculants with active ingredients from living organisms that can mobilize, facilitate, and increase the availability of nutrients from unavailable forms to available forms through mineralization and decomposition processes (Daniel et al., 2022; Chaudhary et al., 2022)

Biofertilizer play important role in soil fertility, improving soil health, and promoting plant growth. and increased crop production (Kumar et al., 2022). Microorganisms contained in biofertilizer will decompose organic matter so that it can be available to plants (Chaudhary et al., 2022). The use of biofertilizer can be applied with the use of microbes, one of which is using phosphate solubilizing bacteria (PSB). PSB is bacteria that plays a key role in the transformation of phosphorus (P) elements that can increase the availability of phosphate in the soil by converting organic compounds into inorganic ones, as well as mineralizing organic compounds by releasing inorganic phosphate (Martaguri, 2009). PSB releases organic acids that can form stable complexes with P-binding cations in the soil so that they can dissolve P from previously unavailable plants to become available (Whitelaw, 2000). PSB also produces growth hormones that play a role in accelerating plant growth and development (Fitriatin et al., 2020). The characteristics of superior isolates of PSB include being able to produce phosphatase enzymes, producing various kinds of organic acids, being able to produce growth hormones to stimulate root and plant growth, and being able to streamline the need for organic fertilizers.

This research was conducted to study the ability of superior phosphate solubilizing bacteria in increasing the growth of rice seedlings in saline media. The results of this study are expected to obtain superior isolates of halotolerant phosphate solubilizing bacteria that can be used as biofertilizers on saline soils.

Materials and Methods

Isolation and Screening PSB

Seven isolates of PSB were isolated from saline soil Cimrutu Village, Patimuan District, Cilacap Regency, Central Java, Indonesia. PSB were isolated from five ecosystems, namely A= rice (*Oryza sativa* L.), B = fallow ecosystem of purun rat (*Eleocharis dulcis*), C = rice field ecosystem (*Oryza sativa* L.) and lotus plant (*Nympheae* sp.), D = rice water fallow ecosystem (*Monochoria vaginalis*), and E = wild grass fallow ecosystem (*Cyperiusiria*).

The serial dilution plate method was used for isolation of PSB using Pikovskaya media. Furthermore, isolates were observed to determine the clear zone of colony diameter and the solubilizing index (SI) was calculated by the formula (Premono et al., 2007):

$$SI = \frac{\textit{Halozone diameter} + \textit{Colony diameter}}{\textit{Colony diameter}}$$

Biofilm characterization and test

The characterization was carried out by looking at the solubilization index of P, and microscopic observation of the morphology of the isolates which included the shape of the bacteria under a microscope with 1000 times magnification and the type of bacteria using the Gram staining method. In the solubilization index of P, the highest value was taken for the biofilm test.

The biofilm test was carried out qualitatively with the Microtiter Plate Assay (MPA).The isolates tested for biofilm were 7 selected isolates produced from the phosphate solvent index test. Preparation was done by inoculating isolates into liquid NA (Nutrient Agar) media. Biofilm staining was carried out by removing planktonic cells by inverting the microtiter plate. The plate wells that give a purple color to the walls indicate the presence of bacteria that can form bacteria (O'Toole, 2011).

Bioassay of 7 selected isolates of phosphate solubilizing bacteria on rice plants in liquid culture media

The design of bioassay used was a Randomized Block Design with seven treatments was repeated three times and used a salinity of 4 dS/m. i.e. control (without PSB), eleven isolates of PSB. The indicator used in the bioassay is rice (Inpari 34) which uses Murphy liquid culture media. Rice seeds were sterilized using 0.2% $HgCl_2$ for ±2 min and 70% alcohol for ±1minute, then the rice was rinsed using distilled water. Sterile seeds were germinated on sterile straw paper for 3 days. Sprouts were planted in 100 mL test tubes containing 95 mL of Murphy's medium and 5 mL of PSB suspension.

IAA Phytohormon Test (Indole Acetic Acid) from 3 superior isolates of phosphate solubilizing bacteria

The IAA Phytohormon test was performed in vitro using the Mac Farland method. speed of 100 rpm for 6 days on a shaker (Ahmad et al., 2005). After the culture liquid has been in the shaker the liquid is centrifuged at 5500 rpm for 10 min. The supernatant was then tested for the ability to produce IAA using the calorimetric method with the addition of the Salkowski reagent. Then it was allowed to stand for 20 min and the absorbance was measured using a spectrophotometer with a wavelength of 535 nm (Patten & Glick, 2002).

Phosphatase enzyme test from 3 superior isolates of phosphate solubilizing bacteria

The analysis of the phosphatase enzyme was determined based on the Eivazy and Tabatai method, namely by giving the substrate p-nitrophenylphosphate so that the p-nitrophenol compound formed due to enzyme activity was then stained with a hydroxide solution that could be calibrated with a 400 nm spectrophotometer (Margesin, 1996).

Production test of organic acid types from 3 superior isolates of phosphate solubilizing bacteria

Organic acid production test using HPLC (High-Performance Liquid Chromatography) method. The filtrate used to obtain organic acid levels are citric, acetic, oxalic, lactic, malic, and ascorbic acids.

Results and Discussion

Biofilm characterization and test

The results of the characterization of the fifteen isolates showed that bacteria were Gram negative and Gram positive (Table 1). Gram negative is characterized by red-colored cells, while Gram positive is characterized by blue to purplish blue cells.

In Table 1, the phosphate solubilization index obtained ranges from 2.05 to 2.83. The highest solubilization index of P in this study was owned by isolate D1 with a value of 2.83, while the lowest solubilization index of P was owned by isolate A3 with a value of 2.05. The difference in solubilization index of P value was related to the ability of each isolate to dissolve bound phosphate. Widawati & Suliasih (2006) stated that each bacterial species has a different ability to produce organic acids, both in number and type during growth, so that it affects the dissolution of phosphate.

Based on Figure 1, it is known that seven isolates produced biofilms as indicated by the presence of a purple dye on the walls of the microtiter plate. According to Robika et al. (2022), the biofilm-forming activity of these bacterial colonies was indicated by the presence of a purple color

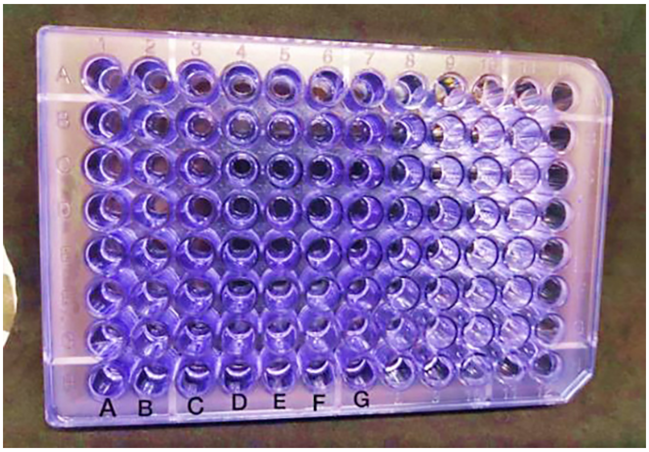

Fig. 1. The qualitative capability to form the biofilm of isolates.
A = A_2, B = A_3, C = C_2, D = D_1, E = D_2, F = D_3, G = E_2

Table 1. Characteristics and Solubilizing Index of Phosphate

Isolate Code	Bacterial Shape	Bacteria Cells Color	Gram Test	Solubilization Index of P
A_1	Cocci	Violet	+	2.143
A_2	Bacilli	Red	–	2.833
A_3	Cocci	Violet	+	2.875
B_1	Bacilli	Red	–	2.067
B_2	Bacilli	Violet	+	2.133
B_3	Bacilli	Red	–	2.217
C_1	Cocci	Red	–	2.105
C_2	Bacilli	Red	–	2.5
C_3	Bacilli	Red	–	2.125
D_1	Bacilli	Red	–	2.428
D_2	Bacilli	Red	–	2.428
D_3	Bacilli	Violet	+	2.727
E_1	Bacilli	Red	–	2.095
E_2	Bacilli	Red	–	2.294
E_3	Bacilli	Red	–	2.166

Note: Isolate A (PSB from the rice field ecosystem); Isolate B (PSB from the fallow ecosystem of the purun rat plant); Isolate C (PSB from rice field ecosystem and lotus plant); Isolate D (PSB from the rice water fallow ecosystem); Isolate E (PSB from the fallow nut grass ecosystem)

Table 2. Effect isolate inoculation on plant height and roots length of rice seedling after 21 days

Selected Isolate Code	Root height, cm	Rooth increment, %	Plant height, cm	Plant increment, %
Control	5.763 a	–	5.437 a	–
PSB 1	8.357 d	45.01	6.991 a	28.58
PSB 2	7.830 cd	35.56	6.420 a	18.07
PSB 3	7.927 ab	37.54	7.817 a	43.77
PSB 4	7.137 bc	23.84	7.487 a	37.70
PSB 5	6.413 cd	11.27	7.620 a	40.15
PSB 6	7.630 cd	32.39	8.837 a	62.53
PSB 7	7.357 bcd	27.65	6.834 a	25.69

Note: PSB 1 = A_2, PSB 2 = A_3, PSB 3 = C_2, PSB 4 = D_1, PSB 5 = D_2, PSB 6 = D_3. PSB 7 = E_2. The average value followed by the same letter in the same column shows no significant difference at the 5% level according to Duncan's Test

attached to the wall of the microtiter plate. The purple color that is not washed or the amount of bound dye is assumed to be the same as the amount of microbial biofilm in the well (O'Toole, 2011).

Based on Table 2, PSB isolate inoculation had a significant effect on root length but had no significant effect on crown height. The difference in rice growth shown in Figure 2, between those treated with isolates and those not treated with isolates was caused by the stimulation of phytohormones.

The length of the plant roots will be more decisive in the absorption of nutrients than the weight of the roots. This is because long roots will be wider in the absorption of nutrients in the soil (Asova et al., 2018). The research of Fitriatin et al. (2022) also showed that using phosphate solubilizing bacteria treatment could increase root length compared to those not using PSB treatment.

Fig. 2. The Effect of PSB isolates on the growth of rice seedlings at 21 days of age

Based on Table 3, root dry weight and shoot dry weight showed that each isolate was able to increase root and shoot dry weight but did not show a significant difference. Root dry weight with PSB 1 treatment had a high tendency increase of 14 µg. The increase in dry weight was due to the activity of PSB media, which acts as a nutrient provider and as a growth promoter (Minaxi et al., 2011). However, root weight plays a less important role in plant nutrient absorption compared to root length (Asova et al., 2018).

Salinity can result in reduced photosynthetic rate, stomata conduction and osmotic inhibition (Mazher et al., 2007; Kabir et al., 2004). Osmotic inhibition occurs when groundwater contains high concentrations of salt and can reduce the ability of plants to absorb water and result in slow growth. So that the slow growth can reduce the dry weight of the plant (Puspafirdausi et al., 2017).

IAA Phytrohormone test (Indole Acetic Acid)

Isolates tested for the content of the IAA hormone produced different results. Table 4 shows that PSB 3 isolates are considered to be the most potential in producing IAA. The amount of concentration produced by the isolate indicates

Table 3. Effect of isolate on plant dry weight at 21 days age

Selected Isolate Code	Root dry weight, µg	Shoot dry weight, µg	Biomass Total, µg	Increment, %
Control	6.00 a	7.00 a	13.00	-
PSB 1	14.00 d	8.00 a	22.00	69.23
PSB 2	10.70 bc	8.00 a	18.70	43.84
PSB 3	13.00 cd	7.70 a	20.70	59.23
PSB 4	8.30 ab	8.00 a	16.30	25.38
PSB 5	12.00 cd	8.00 a	20.00	53.84
PSB 6	10.30 bc	10.00 b	20.30	56.15
PSB 7	12.30 cd	7.70 a	20.00	53.84

Note: PSB 1 = A_2, PSB 2 = A_3, PSB 3 = C_2, PSB 4 = D_1, PSB 5 = D_2, PSB 6 = D_3, PSB 7 = E_2. The average value followed by the same letter in the same column shows no significant difference at the 5% level according to Duncan's Test that the isolate can synthesize the IAA hormone The ability to synthesize tryptophan into IAA was faster in PSB 3 isolates at 4.913 ppm.

The difference in the ability of the IAA hormone produced by the isolates was thought to be due to the difference in the ability of the bacteria to synthesize tryptophan into IAA. The growth hormone produced by phosphate solubilizing bacteria is in the form of auxin which functions in lengthening the roots so that the roots can be longer and stronger in absorbing nutrients. Bacteria that produce IAA will stimulate the growth of the host root system (Herlina et al., 2017). The growth hormone IAA produced by PGPR functions as a signaling molecule that is important in the regulation of plant development stimulates root development of host plants, increases plant resistance to pathogens, and stimulates plant growth (Shaharoona et al., 2006).

Table 4. The ability of PSB isolates to produce IAA phytohormones

Selected Isolate Code	IAA Mean (ppm) and Standard Deviation
Control (Standard Solution)	0.000
PSB 1	4.611 ± 0.043
PSB 3	4.913 ± 0.022
PSB 6	3.819 ± 0.018

Note: PSB 1 = A_2,PSB 3 = C_2, PSB 6 = D_3

Production of phosphatase enzymes and organic acids

Table 5 shows that PSB isolates that have a high ability to produce phosphatase enzyme production are PSB 1 isolates at 3.508 g pNP/g/h, followed by PSB 3 at 3.307 g pNP/g/h and PSB 6 at 3.257 g pNP/g /h. The characteristics of the ability of P solubilizing bacteria isolates can be seen in the ability to produce phosphatase enzymes and the production of various types of organic acids.

Table 5. The ability of PSB isolates to produce phosphatase enzyme

Selected Isolate Code	Phosphatase (µg pNP/g/h) + Standard Deviation
PSB 1	3.508 ± 0.094
PSB 3	3.307 ± 0.012
PSB 6	3.257 ± 0.022

Note: PSB 1 = A2, PSB 3 = C2, PSB 6 = D3

The phosphatase enzyme is an enzyme that will be produced when the availability of phosphate is low (Lidbury et al., 2022). According to Alori et al. (2017), the value of the phosphatase enzyme activity produced is influenced by phosphate solubilizing bacteria that work actively in hydrolyzing organic P. Phosphatase activity works in tandem with the amount of organic P, the high value of phosphatase activity is thought to be because phosphate rhizobacteria work actively to hydrolyze organic P (Amri et al., 2022).

Table 6 shows the results of the analysis that all isolates were able to produce organic acids, namely citric acid, acetic acid, oxalic acid, lactic acid, malic acid, and ascorbic acid with different contents. PSB 1 isolates produced high malic acid at 9.049 ppm compared to other isolates, PSB 3 and PSB 6 isolates produced high lactic acid compared to PSB 1 isolates, namely PSB 3 at 21. 878 ppm and PSB 6 isolates at 7.800 ppm.

The mechanism of phosphate solubilization is closely related to the release of organic acids by phosphate solubilizing bacteria that form chelates with cations (Al, Fe, or Ca) that bind P through hydroxyl and carboxyl chelation, thereby converting them to available forms (Atekan et al., 2014). According to Zhang et al. (2021), Organic acid affect soil phosphorous activation and the ability of organic acids to dissolve phosphate is citric acid > oxalic acid = tartaric acid = malic acid > lactic acid = formic acid = acetic acid (Ryan et al., 2001).

Conclusion

Based on the experiments, it was concluded that a superior isolate of phosphate solubilizing bacteria had been obtained from saline soil which were capable of producing phytohormones, phosphatase enzymes and organic acids. Three superior isolates PSB 1, PSB 3, and PSB 6 produced phosphatase enzymes (3.508 µg pNP/g/h, 3.307 µg pNP/g/h, and 3.257 µg pNP/g/h), IAA (4.611 ppm, 4.913 ppm, and 3.819 ppm), and organic acids (citric, acetic, oxalic, lactic,

Table. 6 Ability of Phosphate Solubilizing Bacterial Isolates to Produce Organic Acid

Selected Isolate Code	Citric (ppm)	Acetic (ppm)	Oxalic (ppm)	Lactic (ppm)	Malic (ppm)	Ascorbic (ppm)
PSB 1	2.294	7.543	3.671	1.443	9.049	2.375
PSB 3	3.884	1.105	2.943	21.878	2.918	1.097
PSB 6	4.413	2.100	2.555	7.800	1.134	2.640

Note: PSB 1 = A_2, PSB 3 = C_2, PSB 6 = D_3

malic and ascorbic). These isolates could form biofilms. Inoculation of PSB isolates affected increasing rice growth, especially on root length. These isolates were able to increase root length by 45.01%, 37.54%, and 32.65%. There isolate were also able to increase plant height by 28.58%, 43.77%, and 62.69%. Superior phosphate solubilizing bacteria , have the potential to be developed as phosphate biofertilizers for rice plants in saline soil.

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