

Exploration and selection of salt tolerant phosphate solubilizing bacteria from the Indonesian saline soils

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Abstract. The constraint on saline land use is the high salt content. Salinity results in inhibition of plant growth. Increasing the productivity of saline land needs to be supported by information on soil bacteria that can be done by exploring phosphate solubilizing bacteria (PSB). Phosphate solubilizing bacteria are non-pathogenic bacteria that are capable of dissolving P not available into a form available for absorption by plants. This study aims to obtain phosphate solubilizing bacteria that are resistant to the highest salinity levels. Soil sampling was carried out in saline land in the North Coast of Central Java, Indonesia. Isolation of phosphate solubilizing bacteria was carried out by pour plate method on pikovskaya selective media. The isolation results obtained 8 pure PSB isolates consisting of BPF RBG2, BPFPT2, BPFDMK1, BPF PKL2, BPF SMG2, BPF BTG2, and BPF KDL2 isolates. PSB isolates were examined microscopically and macroscopically. BPF isolates with the highest resistance to salinity (10.000 ppm NaCl) were BPF BTG2 isolates followed by BPF KDL2, BPF SMG2, and BPF PKL2 isolates.

Key words: Phosphate solubilizing bacteria (PSB), salinity, soil bacteria

1. Introduction

Indonesia is a country that has a fairly large saline area. The total area of saline land in Phosphate solubilizing bacteria saline land of 140,300 ha [1]. Utilization of saline land, especially in the North Coast of Central Java, has not been optimally utilized. High salinity in the soil causes poor absorption of nutrients and shows signs of salt contamination, characterized by leaf yellowing, drying out and plant death [2]. Salinity that occurs in land in the North Coast of Central Java is caused by the entry of sea water through the soil surface or seepage [3]. Increasing productivity of saline land needs to be supported by information about soil bacteria that can be done by exploring phosphate solubilizing bacteria.

Phosphate solubilizing bacteria (PSB) are soil bacteria that are able to increase nutrient absorption of P. Phosphorus (P) is one of the most essential for plant growth, the second being nitrogen. Approximately 95-99% of phosphorus in soil is present in the form of insoluble phosphates [4], but only 0.1% of the total phosphorus exists in plant-accessible form [5]. This problem may be alleviated by releasing P from an immobilized form to a soluble form through the activities of solubilization and mineralization by soil microorganisms [6].

Recently, plant growth-promoting rhizobacteria (PGPR) have become attractive for use as soil inoculums due to their improvement of plant growth and yield by various direct or indirect mechanisms [7, 8]. Among PGPR, PSB are one of the most interesting microorganisms concerned with plant P nutrition. The principal mechanism for mineral phosphate solubilization by these bacteria is the production of low-molecularweight organic acids, which have high potential as cations bound to phosphate, as a result of their conversion into soluble forms [6]. These bacteria are able to break down organic P into inorganic P by secreting organic acids such as formic acid, fumarate, succinate, lactate, acetate, glycolic, and propionate, so they can also reduce the pH of soil [9]. PSB can dissolve P ions bound by soil cations such as Fe, Ca, Mg, and Al which are then converted into forms available for absorption by plants. These bacteria can dissolve bound P ions because it produces the enzyme phosphatase. In general, the growth of phosphate solubilizing microorganisms is influenced by soil acidity. Indeed, the growth of these microorganisms is in line with the increasing acidity of the soil. Phosphate solubilizing bacteria can grow optimally in the pH of 4 - 10.6 [10].

PSB has the ability to dissolve tricalcium phosphate from an insoluble form into a soluble form, as has been reported by many researchers [11, 12, 13, 14]. Additionally, the use of PSB as inoculants simultaneously increases P uptake by the plant, resulting in higher crop yields - as has been documented in maize [15], green beans [16], sorghum [17], wheat, potato, bean [18] and tomato [19]. The purpose of this study was to obtain PSB that was resistant to high salinity levels for an inoculum-making material that can be easily applied to plants in saline soil. The benefit of this research was to provide information on PSB which have the highest salinity resistance which can be used as a material for making saline-resistant inoculums that can be applied to plants in saline fields.

2. Materials and methods

The material used was saline soil taken from 8 regencies along the North Coast of Central Java, Indonesia including Demak, Semarang, Pati, Rembang, Kendal, Batang, Pekalongan, and Pemalang, pikovskaya media (composition per 1 liter of aquadest: 10 g glucose, 0.2 g KCl, MgSO₄ 0.1 g, MnSO₄ 0.1 g, FeSO₄ 0.1 g, Ca₃ (PO₄)₂ 5 g, (NH₄)₂SO₄ 0.5 g, Agar 15 g), and NaCl.

2.1. Soil Sampling

Soil sampling was carried out in saline land in the North Coast of Central Java. Soil samples were taken to a depth of about 20 cm from the soil surface. The soil sample was then measured for pH, EC, TDS, and NaCl levels.

2.2. Isolation of Phosphate Solubilizing Bacteria

A 25 g soil sample was suspended with 225 ml sterile aquadest in an Erlenmeyer flask. The soil sample suspension is then shaken for about 30 minutes (10¹ dilution). The following series of dilutions, namely from the previous dilution, 1 ml was taken and added to 9 ml of sterile distilled water then homogenized (10² dilution), and so on. A total of 1 ml of soil suspension from a 10² dilution was put into a petri dish using the pour plate method. The petri dishes were then closed tightly and incubated at room temperature at 37°C for 3 - 4 days. Phosphate solubilizing bacteria was indicated by the presence of colonies that have clear zones (hallozone) on pikovskaya media. Colonies that had clear zones were then grown on NA slanted media to obtain pure cultures and used as stock for further testing.

2.3. Identification of Phosphate Solubilizing Bacteria

Phosphate solubilizing bacteria were identified by macroscopic and microscopic observations. Macroscopic observations were carried out by observing the phosphate solubilizing bacteria on the visible parts of the colony shape, surface, elevation, colony edges, and colony color. Microscopic observation was carried out by using gram stain and observing the shape of the cells under a microscope.

2.4. Resistance Test of Phosphate Solubilizing Bacteria to Salinity

The resistance test of phosphate solubilizing bacteria to salinity was carried out on pikovskaya media with the addition of NaCl in accordance with the salinity level treatment. Phosphate solubilizing bacterial isolates were inoculated on pikovskaya media using the spread plate method. Then it was incubated at room temperature for 3 - 4 days. The phosphate solubilizing bacteria colonies that were able to grow on pikovskaya media with this salinity level were then counted the number of colonies.

2.5. Increasing the resistance of phosphate solubilizing bacteria to salinity

Increasing the resistance of phosphate solubilizing bacteria to salinity was carried out by growing the phosphate solubilizing bacteria on pikovskaya media whose NaCl levels were increased gradually, so that phosphate solubilizing bacteria were obtained that were resistant to the highest salinity. Bacterial colonies that were still growing on pikovskaya media with high salinity levels showed their resistance to high salinity levels. The data analysis was done quantitatively.

3. Results and Discussion

3.1. Soil Analysis of the North Coast of Central Java, Indonesia

Saline soil originated from eight regencies along the north coast of Central Java, Indonesia including Rembang, Pati, Demak, Semarang, Kendal, Batang, Pekalongan, and Pemalang. The eight regencies represent areas with climate types of C, D, and E in the climate classification according to Oldeman. Oldeman is a regional climate classification method based on the yearly rainfall indicators. The regional climate classification is based on the information regarding the number of wet and dry months according to the intensity of yearly rainfall of an area. Oldeman classified an area into five major types namely A, B, C, D, and E and four sub-types of climate. Pemalang, Pekalongan, Batang, Kendal, and Semarang have climate types C. Demak and Pati have climate types D, while Rembang has climate types E. According to the Oldeman climate classification, an area with climate type C is an area that has 5-6 consecutive wet months. Areas with climate type D have a number of consecutive wet months of 3-4 months, while regions with climate type E only have wet months of less than 3 consecutive months. This shows that the area with climate type E is a dry area. The results of analysis of the soil samples which include the values of EC (Electrical Conductivity), TDS (Total Dissolved Solids), NaCl content, and soil pH were listed in Table 1.

Table 1. Results of soil pH, EC, TDS, and NaCl analysis in the North Coast of Central Java

No.	Soil samples location	pH	Temp. (°C)	EC (mS/cm)	Temp. (°C)	TDS (g/l)	Temp. (°C)	NaCl (%)	Temp. (°C)
1	Pati 1 (-)	7.66	28.2	16.21	28.5	8.15	28.7	32.5	28.6
2	Pati 2 (+)	7.69	28.0	7.29	28.2	3.63	28.5	13.7	28.4
3	Rembang 1 (-)	8.20	27.9	74.70	28.2	37.90	28.2	148.8	28.6
4	Rembang 2 (-)	7.80	27.8	17.71	28.3	8.85	28.3	34.8	28.2
5	Rembang 3(+)	7.55	27.8	11.53	28.8	6.10	28.5	20.8	28.8
6	Demak (-)	6.98	29.6	23.04	29.6	10.83	29.8	40.3	30.3
7	Batang 1 (-)	4.69	29.2	14.20	29.2	6.94	29.2	23.7	29.2
8	Batang 2 (+)	4.77	28.6	10.18	29.4	4.79	29.2	18.6	29.2
9	Kendal 1 (-)	3.53	27.8	8.35	27.9	4.62	28.2	15.8	28.1
10	Pemalang 1 (-)	4.63	27.5	32.4	27.7	16.70	27.7	63.5	27.7
11	Pemalang 2(+)	4.95	27.5	16.92	27.8	8.44	27.7	34.1	27.7
12	Pekalongan 1 (+)	4.81	28.6	9.47	29.9	4.56	29.6	17.3	29.8
13	Pekalongan 2 (-)	7.69	28.0	66.30	28.9	35.50	28.2	131.3	27.9
14	Semarang 1(+)	6.88	28.6	12.31	29.4	6.13	29.5	24.1	29.4
15	Semarang 2 (-)	7.88	28.6	9.12	29.2	4.69	29.3	17.8	29.1

Information: (-) = without vegetation, (+) = with vegetation

Table 1 shows that the land in the north coast of Central Java, with a distance of about 4 m to 1 km from the shoreline, had moderate to very high salinity levels. The salinity level is divided into 5 categories based on the DHL value in mS / cm, namely very low (0 - 2), low (2 - 4), medium (4 - 8), high (8 - 16) and very high (> 16). Soil salinity levels usually change according to the season. Soil sampling in this study was carried out in the dry season in August-September 2020. The level of soil salinity usually increases during the dry season, because the amount of water that comes from rain is not enough to neutralize the amount of water lost by evapotranspiration, so that when water is evaporated into the atmosphere it causes soluble salts accumulate to the soil surface [20]. The causes of soil to become saline are (1) the land has parent material containing salt deposits [21] (2) sea water intrusion, salt accumulation from irrigation water used or groundwater movement reclaimed from the seabed [20], (3) high evapotranspiration rate with low rainfall so that minerals are not washed completely [22].

The highest soil salinity level was achieved by Rembang with soil samples without vegetation that had an electrical conductivity value EC 74.7 mS / cm. Rembang has a climate type E which only gets less than 3 consecutive wet months for 1 year. The area with climate type E is a dry area. Saline soils are found in dry area with an average rainfall of less than 500 mm / year [21]. The amount of water that comes from rain is not sufficient to neutralize the amount of water lost by evaporation and evapotranspiration, so when water is evaporated into the atmosphere it causes soluble salts to accumulate to the soil surface and cause the soil to become saline.

The EC value of soils overgrown with plants shows a lower level of salinity while bare soil had a high level of salinity. This shows that soil with very high salinity is difficult for plants to grow. Soils containing alkaline salts can have a toxic effect and increase the osmotic pressure of the roots thereby inhibiting plant growth [23].

3.2. Isolation and Identification of Phosphate Solubilizing Bacteria

The isolation of phosphate solubilizing bacteria from the soil resulted in 8 types of isolates, namely BPF RBG2, BPFPT2, BPFDMK1, BPF PKL2, BPF SMG2, BPF BTG2, and BPF KDL2. The presence of BPF isolates was indicated by the presence of a clear zone (halozone) around the colony. The isolates obtained were tested for identification of Gram stain, morphological and microscopic form of the isolates that have been obtained, as in Table 2.

Table 2. Gram Staining, Morphological and Microscopic characteristics of Phosphate Solubilizing Bacteria Isolates

No	Isolates name	Gram stain	Colony shape	Surface	Elevation	Edge colony	Colony color	Cell shape
1.	BPFPT2	Positive	Irregular	Unshine	Flat	Undulate	Yellowish white	Coccus
2.	BPF RBG2	Negative	Irregular	Unshine	Raised	lobate	Yellowish white	Basil
3.	BPFDMK1	Positive	Circular	Unshine	Raised	Undulate	Yellow	Basil
4.	BPF BTG2	Positive	Circular	Unshine	Convex	entire	White milk	Basil.
5.	BPF KDL2	Positive	Circular	Unshine	Raised	entire	Yellow	Coccus
6.	BPF PML2	Positive	Circular	Unshine	Flat	entire	Yellow	Basil
7.	BPF PKL2	Positive	Irregular	Unshine	Flat	lobate	Yellow	Coccus
8.	BPF SMG2	Positive	Circular	Unshine	Convex	entire	Yellow	Basil

One method that is still needed in bacterial taxonomy is Gram stain [24]. This method is used to separate the members of the bacterial domain into two groups based on their cell walls. Gram positive bacteria have a simpler cell wall, with relatively large amounts of peptidoglycan. The cell wall of gram-negative bacteria has less peptidoglycan and is structurally more complex [25]. Gram-negative bacteria are characterized by cells that are red in color and gram-positive bacteria in purple [26].

3.3. Resistance of Phosphate Solubilizing Bacteria to Salinity

Phosphate solubilizing microorganisms can be isolated from soils with low phosphate content, especially in the area around plant roots, because these bacteria use small amounts of phosphate and are able to utilize inorganic phosphate which is not available for metabolic purposes. To detect and estimate the ability of phosphate solubilizing microorganisms, a pour plate method was used.

The selective media commonly used to isolate and multiply phosphate solubilizing organisms is Pikovskaya agar [27]. The medium is cloudy white, because it contains insoluble phosphates such as calcium phosphate. After 3-4 days of incubation, the potential for microorganisms to dissolve phosphate

is characterized by a clear zone (halozone) around the colony. The ability of each phosphate solubilizing microorganism to grow and dissolve phosphate is different. The superior phosphate solubilizing microorganisms will produce the largest halozone diameter compared to other colonies [28].

Phosphate solubilizing bacteria of genus *Pseudomonas* and *Bacillus* can survive in saline soil conditions [29]. Yuliani and Rahayu [30]'s research showed that the use of *Pseudomonas* resulted in more optimal growth than the use of *Bacillus* in saline media. Test the resistance of saline-resistant phosphate solubilizing bacteria as in Table 3.

Table 3. Numbers of colonies (log CFUx10⁶) Isolates of Phosphate Solubilizing Bacteria on Various Salinity of Pikovskaya Media (ppm NaCl)

No	Isolate	Media Salinity Level (ppm NaCl)										
		0	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000
1.	BPFPT2	2,01	1,41	1,36	1,11	1,52						
2.	BPFBRG2	2,20	1,95	1,36	1,20	1,38	1,53	1,58	1,60	1,62	1,67	
3.	BPFDMK1	1,11	1,54	1,91	1,26	1,74	1,18					
4.	BPFBTG2	1,76	1,49	1,42	1,40	1,04	1,65	1,34	1,60	1,30	1,81	2,09
5.	BPFKDL2	0,90	0,69	2,52	1,58	1,32	1,34	1,15	2,05	1,04	1,04	1,60
6.	BPFPM2	0,85	1,70	1,54	2,20	1,58						
7.	BPFPKL2	1,36	1,38	1,15	1,15	1,81	1,23	1,28	1,26	1,32	1,52	0,90
8.	BPFSGM2	1,62	1,74	1,26	2,03	1,52	1,67	1,63	1,85	1,77	1,70	1,08

Table 3 shows that phosphate solubilizing bacterial isolates were obtained that were resistant to very high salinity levels reaching 10,000 ppm NaCl. BPFBTG2 isolates showed the highest level of resistance to salinity followed by BPFKDL2, BPFSGM2, and BPFPKL2 isolates. The number of colonies on media with increased salinity content sometimes increased the number of colonies. This was probably because the phosphate solubilizing bacteria has begun to tolerate high salinity levels. Bacteria that were grown at a low salinity level, then grown again at a salinity level which gradually increased in salinity, would also show their resistance to high salinity levels to a certain extent. The resistance of phosphate solubilizing bacteria obtained at high salinity levels was also thought to be caused by the origin of the isolates taken from coastal areas. The saline-resistant isolate obtained was as shown in Illustration 1.

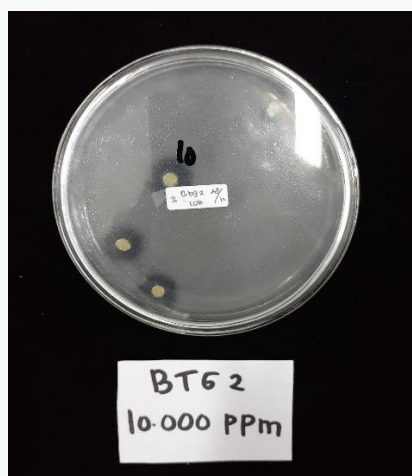


Illustration 1. The saline-resistant isolate

There are microorganisms that are tolerant of high salt levels called halotolerants and there are microorganisms that require high salt concentrations called halophilics. The phosphate solubilizing bacteria obtained may include halotolerants that were still able to survive at high salt concentrations. Apart from affecting the osmotic pressure, high salt concentrations tend to change the properties of proteins by disrupting the protein structure which is important for enzymatic activity. The resistance mechanism of halotolerant microorganisms is that they tend to excrete relatively high and relatively toxic concentrations of sodium ions from the inside of their cells to achieve osmotic equilibrium [31].

4. Conclusion

From the results of the study, it could be concluded that there were 8 kinds of phosphate solubilizing bacterial (PSB) isolates including isolates BPFRBG2, BPFPT2, BPFDMK1, BPFPKL2, BPFMSG2, BPFBTG2, and BPFKDL2. PSB isolates with the highest resistance to salinity (10,000 ppm NaCl) were BPFBTG2 isolates, followed by BPFKDL2, BPFMSG2, and BPFPKL2 isolates.

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