

In vitro selection of rock phosphate solubility by microorganism from Ultisols in South Sulawesi, Indonesia

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Abstract: Phosphorus (P) is the second most required nutrient of plants, but its availability in ultisols is relatively low. Source of P can be found from rock phosphate (RP) which has low solubility level. Use of microbial solubilizing phosphate such as bacteria, fungi or combination of both is potential to increase availability of P. This research was aimed to find bacterial or fungal isolates that are high potential in solubilizing of RP. In vitro selection was conducted to obtain indigenous bacteria or fungi from Ultisols of South Sulawesi. Selection of microbes for their capability to solubate rock phosphate qualitatively, conducted through measuring the solubility index (SI) and solubility efficiency (SE) on pykovskaya solid medium and on National Botanical Research Institutes phosphate-bromophenol blue (NBRI-PBP) medium. The results of this study indicated that from SI and SE highest values, four high potential isolates were selected namely, *Aspergillus niger* JTM 2, *Bacillus* sp JTM 3, *Aspergillus niger* JTM 6 and *Pseudomonas* sp JTM 10. Ability of solubilizing phosphate from the four isolates was then tested in liquid medium pikovskaya either in single or mixed combination. Solubilizing phosphate of RF was determined according to [1] method. The results of test on solid media indicate that *Bacillus* sp JTM 3 and *Pseudomonas* sp JTM 10 have SI and SE higher than both *Aspergillus niger* JTM 2 and *Aspergillus niger* JTM 6. Furthermore, test on Pikovskaya liquid media confirmed that *Aspergillus niger* JTM 6 and *Pseudomonas* sp JTM 10 gives the higher results than other single isolates and control, whereas the combination of isolates *Bacillus* sp JTM 3 and *Aspergillus niger* JTM 2 indicated significantly higher solubilizing phosphorus production than all other treatments.

Keywords: In Vitro, Mixture Isolates, Pikovskaya Media, Rock Phosphate, Ultisols

1. Introduction

In Indonesia dry land which has acid reaction such as Ultisols, Oxisols and Inceptisols covers a total area of 65.49% (102.8 million ha). Ultisols soil is very potential to be used to increase food production due to its spread about 28.32% of available dry land [2]. The main characteristics of Ultisols are acid soil reaction (pH), cation exchange capacity, base saturation and low C organic, aluminum content (Al), iron (Fe) and manganese (Mn) and high phosphorous (P) fixation [3,4]. High fixation of P causes the element not available for plant. The existence of various constraints in the Ultisol soil can decrease real production.

Phosphor (P) is the second nutrient element after nitrogen mostly required by plant. P is the main component of nucleic acid, phospholipid, ATP and some coenzymes. The effectiveness of using of phosphate fertilizer is very low only about 15% - 20% [5] and 10 – 25% [6].

Rock phosphate (RP) as a source of P is not expensive, but the availability of P is low. Solubility of RP can be increased by phosphate solubilizing microorganism (PSM). Increasing RP solubility by microorganism is due to the lowering of pH and or organic acid excretion [7, 8]. *Pseudomonas putida* and *Serratia mesenteroides* were reported to increase solvable P at RP 6 – 19 times [9]. The ability of effective *Pseudomonas* and *Bacilli* in dissolving phosphate was also put forward by [10]. RP solubility is 10 times more was also obtained through the use of *Aspergillus niger* [11,12].

RP solubility mechanism by PSM is due to the effect of lowering pH and excreted organic acid by PSM [8,13]. Other researchers found that the P solubility was not always correlated negatively with pH [14-19]. Alam S., S. Khalil, N. Ayub and M. Rashid [20] found the existence of another factor besides organic acid in the solubility of P. Excreted organic acid can dissolve RP and or chelate cation from P

to dissolve it [21].

Type and quantity of organic acid produced by PSM depends on strain, media composition, growth condition, etc, but most dominant PSM produces citric and oxalic acid [20, 13]. Y. Sastro, D. Widiyanto and D. Shiddieq [22] found that dominant organic acid in *Aspergillus niger* was malate acid following citrate and oxalate. However, there was microorganism that did not produce organic acid but it could dissolve P [23]. Based on various mechanisms in the solubility of P, the ability of PSM in dissolving P is also different. Therefore the solubility of RP by single isolate or mixture of PSM isolates from fungi and bacteria that can synergize is hoped to be obtained in this study so that RP solubility can be more increased.

The main aim of this study is to obtain PSM isolate from Ultisol in Moncongloe which has high potential in dissolving RP through in vitro selection both in NBRIP-BPB solid media and pikovskaya liquid media.

2. Materials and Methods

2.1. Isolation of PSM from Plant Rhizosphere

Isolation of phosphate solvent was taken from the area of grass rhizosphere and cassava in Ultisol at Moncongloe village, Maros regency, Indonesia which is reacted to acid (pH 5.37) having low cation exchange capacity (26.77 cmolkg⁻¹) and very low base saturation (15%) and high Al saturation (24.62%). From the rhizosphere area 10 g of soil was taken and then dissolved in 90 ml sterile distilled water homogenized with vortex. Then 1 ml soil solution was extracted and put into a test tube containing 9 ml sterile distilled water until 10⁻⁵ dilution. The result of the dilution was then planted in pikovskaya solid media. Pikovskaya solid media [24] contained tricalcium phosphate (rock phosphate) 5 g, glucose 10 g, (NH₄)₂SO₄ 0.5 g, NaCl 0.2 g, MgSO₄·7H₂O 0.1 g, KCl 0.2 g, yeast extract 0.5 g, MnSO₄ trace, FeSO₄·7H₂O trace, agar 15 g, then were dissolved in 1000 ml sterile water and incubated for 5 days. This isolate growing colony was reinoculated to be purified and tested further.

2.2. PSM Ability Test to Dissolve Rock Phosphate in Solid Media

Pure PSM was then tested its ability to dissolve P in selected pikovskaya media, but not all PSM growing on the media can produce halozone (clear zone). Therefore the test ability to dissolve P was also done by using media from the National Botanical Research Institutes Phosphate - Bromophenol blue (NBRIP-BPB). NBRIP-BPB media have compositions Ca₃(PO₄)₂ 5 g, glucose 10 g, (NH₄)₂SO₄ 0.1 g, MgSO₄·7H₂O 0.25 g, KCl 0.2 g, MgCl₂ 6 H₂O 5 g, BPB 0.025 g, agar 15 g dissolved in 1000 ml sterile distilled water [25].

In this study the source of phosphate used came from RP containing: P₂O₅ total 30.68%, P₂O₅ available 21.36%, P₂O₅ dissolved citrate acid (2%) 10 – 12%, its smoothness passed

the sieve 250 µm. Ability of PSM dissolving RP was done by placing one ose of cultivated PSM in sterile NBRIP-BPB solid media, then incubated for 1 – 6 days at room temperature (28°C). Observation to established halozone (clear zone) by PSM was started a day after the growing. Ability of PSM in dissolving undissolved P could be known by solubilization index (SI) according to formula: total diameter of halozone divided by colony diameter [26] and solubility efficiency (SE) of halozone diameter divided by colony diameter times 100 [27].

$$SI = \text{halo zone diameter} / \text{colony diameter} \quad (1)$$

$$SE = \text{halozone diameter} / \text{colony diameter} \times 100 \quad (2)$$

2.3. PSM Ability Test to Dissolve Rock Phosphate in Liquid Media

Dissolved phosphates due to PSM activity can be known quantitatively by using pikovskaya liquid media i.e., 25 ml (without agar) using RP (5 g/1000 ml), then was autoclaved at temperature 121°C for 20 minutes. PSM 1 ml (1x10⁸ cfu/ml) was inoculated into each sterile media for *Bacillus* sp (JTM3) and *Pseudomonas* sp (JTM10), whereas *Aspergillus niger* (JTM2 and JTM6) 1 ml (1x10⁶ cfu/ml). Ability test to dissolve P was done both at single isolate and combination between bacterial isolate and fungi (Table 2). Treatment then was placed at shaker for 12 days with rotary shaker 120 rpm. Suspension was filtered with whatman 40 then centrifuged for 15 minutes at 10.000 rpm. Measurement of dissolved P in media was done according to Murphy and Riley [1] using spectrophotometer at the wave length 693 nm calibrated with standard curve KH₂PO₄.

3. Results

3.1. Isolation of PSM and Test on Solid Media

Isolated PSM from rhizosphere of cassava plant, grass and legume from acid soil (Ultisol) at Moncongloe planted in pikovskaya media and NBRIP-BPB (Table 1) shows the growing PSM in pikovskaya media only 2 isolates forming narrow halozone as indicator of the P solubility. In NBRIP-BPB media all growing colony formed halozone of different diameters. Based on the halozone diameter formed in the NBRIP-BPB media, there are 4 potential isolates dissolving high RP.

Table 1. PSM isolated from grass rhizosphere, cassava and legume in Ultisol Moncongloe, South Sulawesi

No. Isolate	Media of pikovskaya		Media of NBRIP-BPB	
	colony	halozone	Colony	halozone
JTM1	+	–	+	√
JTM2	+	√	+	√√
JTM3	+	–	+	√√
JTM4	+	–	+	√
JTM5	+	–	+	√

No. Isolate	Media of pikovskaya		Media of NBRIP-BPB	
	colony	halozone	Colony	halozone
JTM6	+	√	+	√√
JTM7	+	–	+	√
JTM8	+	–	+	√
JTM9	+	–	+	√√
JTM10	+	–	+	√
JTM11	+	–	+	√

Description: + = growing √ = halozone formed – = no halozone formed

In pikovsakaya solid media (Table 1) only 2 isolates formed halozone with small diameter whereas in NBRIP-BPB the formed halozone was clear and had bigger diameter. Based on the formed halozone, there are 4 high potential isolates which can dissolve P: isolate *Bacillus* sp

JTM3, isolate *Pseudomonas* sp JTM10, isolate *Aspergillus niger* JTM2 and isolate *Aspergillus niger* JTM6. Four isolates which have big halozone diameter was then tested in NBRIP-BPB media to measure the solubility index (SI) and the solubility efficiency (SE) toward RP. Halozone diameter from group of bacteria (*Bacillus* sp and *Pseudomonas* sp) formed was smaller (2.10 – 2.23 cm) than the *Aspergillus niger* fungi halozone diameter (6.57 – 8.50 cm) measured at the age of 5 days, but the SI and the SE of bacteria were bigger than the fungi (Table 2).

3.2. PSM Ability Test in Pikovskaya Liquid Media

Ability test in dissolving RP quantitatively from 4 isolates was done by using liquid pikovskaya media containing 5% RP. This observation shows the lowering pH of liquid media inoculated by PSM both in the treatment of single isolate and mixed isolates compared to control pH.

Table 2. Solubility index and solubility efficiency of RP by PSM in NBRIP-BPB media

Microbia	Age of isolate (day)	Diameter of colony (cm)	Diameter of Halozone (cm)	solubility index	solubility efficiency
<i>Bacillus</i> sp JTM3	2	0,95	1,58	1,67	166,67
	3	1,05	1,78	1,70	169,84
	4	1,07	2,00	1,88	187,50
	5	1,10	2,23	2,03	203,03
	6	1,10	2,62	2,38	237,88
	7	1,12	2,73	2,45	244,78
	Average				2,02
<i>Pseudomonas</i> sp JTM10	2	0,70	1,18	1,69	169,05
	3	0,92	1,85	2,02	201,82
	4	0,95	2,00	2,11	210,53
	5	0,98	2,10	2,14	213,56
	6	1,02	2,27	2,23	222,95
	7	1,03	2,42	2,34	233,87
	Average				2,09
<i>Aspergillus niger</i> JTM2	2	2,00	2,75	1,38	137,50
	3	2,50	4,05	1,62	162,00
	4	3,45	5,37	1,56	155,56
	5	4,38	6,57	1,50	149,81
	6	5,23	7,98	1,53	152,55
	Average				1,51
<i>Aspergillus niger</i> JTM6	1	1,20	2,25	1,88	187,50
	2	2,48	3,88	1,57	156,57
	3	3,35	5,30	1,58	158,21
	4	4,35	7,15	1,64	164,37
	5	5,40	8,50	1,57	157,41
Average				1,65	164,81

Higher solubility of RP was not always correlated with lower pH. Isolate of JTM2 (pH 4.01) the dissolved RP was only 19.14 mg P l⁻¹. On the contrary, isolate JTM10 (pH

6.64), the dissolved RP was 76.33 mg P l⁻¹. Correlation between low pH and the RP solubility can be shown by the treatment of mixture of isolate JTM2 and isolate JTM3. The

RP solubility increased clearly due to PSM incubation compared to control (Table 3) except for the treatment of isolate JTM3 and mixture of isolate JTM2+3+6 was not different significantly from the control based on Duncan test at the level of significance 95%. This result of this study shows synergy between isolate JTM2 and JTM3 because the RP solubility of single treatment was only 19.14 mg PI^{-1} and 7.49 mg PI^{-1} respectively, whereas the treatment mixture of isolate JTM2 and dissolved JTM3 RP increased to 87.59 mg PI^{-1} .

Table 3. P solubility from RP and pH change by PSM in Pikovskaya liquid media

No.	Treatments	Average pH liquid media	Average P soluble (mg PI^{-1})
1	Isolate JTM2	4,01	19,14 b
2	Isolate JTM3	5,52	7,49 a
3	Isolate JTM6	5,97	66,47 de
4	Isolate JTM10	6,64	76,33 e
5	Isolate JTM2 + 3	4,74	87,59 f
6	Isolate JTM3 + 6	5,99	32,51 b
7	Isolate JTM2 + 10	5,34	29,12 b
8	Isolate JTM6 + 10	5,90	18,86 b
9	Isolate JTM2 + 3 + 6	6,32	6,57 a
10	Isolate JTM2 + 6 + 10	5,77	68,90 de
11	Isolate JTM2 + 3 + 10	6,19	59,34 c
12	Isolate JTM3 + 6 + 10	6,61	67,23 de
13	Isolate JTM2 + 3 + 6 + 10	6,49	59,40 cd
14	Control (without PSM)	7,16	0,18 a

4. Discussion

4.1. PSM in Solid Media

Based on the selection in pikovskaya solid media, the formed halozone was only small so that it was difficult to identify the formation of halozone clearly from PSM colony. In order to overcome the difficulty, the test was done in NBRIP-BPB media [25]. Nautiyal [28], found that the use of yeast extract in pikovskaya media inhibit the growth of PSM. In the same case based on our experiment, we found that NBRIP-BPB media can form halozone that was clearer. This properties is helpful in identifying PSM that can solubilize phosphate. This one of superiority of NBRIP-BPB media comparing to pikovskaya media.

This observation to colony diameter and halozone diameter in NBRIP-BPB media toward 4 PSM isolates shows *Aspergillus niger* (JTM2 and JTM6) was bigger compared to *Bacillus* sp JTM3 and *Pseudomonas* sp JTM10, but the observation of SI and SE shows *Bacillus* sp JTM3 and *Pseudomonas* sp JTM10 was higher compared to *Aspergillus niger* JTM2 and *Aspergillus niger* JTM6. The average values SI and SE of bacteria were 2.05 and 205.12 respectively, whereas JTM2 and JTM6 were 1.58 and 158.14 respectively. This indicates that the bacteria dissolve higher

P than *Aspergillus*. Ability of bacteria to dissolve higher P than fungi also has been reported before [20, 23].

Observation to SI and SE in solid media was an early selection to microorganism that can dissolve P qualitatively. Therefore further test is still needed in liquid media, because in solid media it has not reflected the power of dissolving P quantitatively. Besides the amount of SI and SE may not reflect the power to dissolve RP in pikovskaya liquid media [29,30]. The same thing happens at the observation of SI and SE in isolate JTM 3 was 2.02 cm and 201.62 but dissolved P in liquid media was only 7.49 mg PI^{-1} . On the contrary, isolate JTM 6 has SI and SE 1.65 and 164.81 but dissolved P was 66.47 mg PI^{-1} .

4.2. PSM in Pikovskaya Liquid Media

Theory of P solubility by PSM in liquid media refers to production of organic acid, lowering of pH and the chelation to cations by organic acid so that P becomes dissolve. This observation shows that all PSM treatments have low pH between 4.01 and 6.64 both in single treatment and mixed treatment; whereas in control (without PSM) the pH was 7.16. Lowering of pH in media in PSM treatment is due to production of organic acid [31] and by PSM respiration [14]. Lowering of pH accompanied by production of organic acid will increase P solubility.

Negative correlation between pH and P solubility in this study can be seen in treatment JTM2+JTM3, JTM2+JTM6+JTM10 and JTM 6 of which the pH was 4.74, 5.77 and 5.97 respectively. Dissolved P was 87.59 mg PI^{-1} , 68.90 mg PI^{-1} and 66.47 mg PI^{-1} respectively. The increase of P solubility was due to the lowering of pH accompanied by organic acid production [13,8]. However, some researchers indicate that there is no correlation between pH and P solubility [14-18]. The same thing is also indicated by this study that is in the treatment of single isolate JTM10 and mixed isolates JTM3+JTM6+JTM10, the pH was 6.64 and 6.61 respectively but the RP solubility was higher than some treatments of low pH. According to Illmer and Schinner [14], P solubility in relatively high pH was due to chelation of organic acid with ion Ca in tricalcium phosphate. Result of this study also shows the treatment of single isolate JTM2 and JTM3 can dissolve RP only 19.14 mg PI^{-1} and 7.49 mg PI^{-1} respectively, but in mixed treatment between isolate JTM2 and JTM3 the RP solubility increased to 87.59 mg PI^{-1} , so there was a synergic effect between the two. Existence of synergy may be caused by resultant between the lowering of pH and production of organic acid due to mixture of isolates JTM3 +JTM2. Mixture of isolates JTM2 and JTM3 caused the lowering of pH of isolate JTM3. Lowering of pH caused the change of organic acid produced was more dominant citrate acid [32]. Citrate acid has power to dissolve P greater than oxalic acid and malate [23].

5. Conclusion

The use of NBRIP-BPB media makes observation of SI and SE values possible and clear compared to pikovskaya

media. The values of SI and SE of *Bacillus* sp JTM3 and *Pseudomonas* sp JTM10 are bigger than *Aspergillus niger* JTM2 and JTM6 planted in NBRIP-BPB solid media. Test of PSM in solid media through observation of SI and SE has not reflected the ability to dissolve P because it is not always compatible with quantitative test in liquid media. In pikovskaya liquid media the single isolate *Pseudomonas* sp JTM10 and mixed isolates between *Aspergillus niger* JTM2 and *Bacillus* sp JTM3 is found to be more effective in dissolving P.

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