Screening of the drought tolerant phosphate solubilizing bacteria in dissolving P-inorganic

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Abstract. The drought tolerant phosphate solubilizing bacteria is needed to dissolve inorganic phosphate (P) which is usually low in the availability at dry land. This study is aimed to obtain a combination of drought tolerant phosphate solubilizing bacteria which has high potential in dissolving P-inorganic. An experimental study which has 4 treatments of bacterial combinations has been conducted in a laboratory. The first treatment was the combination between Pseudomonas azotoformans (A) and Acinetobacter baumannii (B). The second treatment was the combination of A and Bacillus paramycoides (C). The third treatment was B and C, then the fourth treatment was A, B, and C. The potential of the bacterial combination in dissolving P-inorganic was measured qualitatively using phosphate solubilizing index (PSI) on pikovskaya solid medium. While, the potential of the bacterial combination in dissolving P-inorganic was measured quantitatively by measuring dissolved P using the ascorbic acid method in pikovskaya liquid medium containing 0.5% Ca₃(PO₄)₂. The results showed that the combination of those three bacteria (A B and C) has the best potential to dissolve P-inorganic compared to other combinations. In addition, the three bacteria combination also resulted in the highest dissolved P with 0.29% potential dissolution of the total Ca₃(PO₄)₂ contained in the pikovskaya liquid medium, followed by combination of B and C (0.19%), and A and C (0.18%), respectively. Thus, before the combination of these three bacteria is applied in soil, it is needed further experiment of the potential of the bacteria in dissolving soil P-inorganic.

1. Introduction
Phosphorus (P) is the second essential macro nutrient required in the relatively large amount for optimizing the plant growth and yield. Plants need phosphorus not only for developing phospholipids and adenosine three phosphate (ATP) but also for providing energy resource of the photosynthesis, respiration and macromolecular synthesis [1]. In soil, phosphorus is generally immobile and exists abundantly in the form of mineral complex of tricalcium phosphate Ca₃(PO₄)₂ that is very resistant to dissolution [1,2]. Plants absorb phosphate in an orthophosphates ionic forms (H₂PO₄⁻ and HPO₂⁻) that are available in soil solution. In soils, there is an equilibrium process between P bound to soil minerals and P in soil solution. Under normal conditions, the rate of P released from complex of soil mineral is slower than the rate of P absorbed by plants. As a result, the availability of P in soil solution tends to be the condition causing P deficiency for plants. Commonly, it is only 0.1 % of the total phosphorus in soils available for plant [3].
The P availability in soils depends on the solubility rate of P-minerals. Although the phosphate release mechanism of (Ca$_3$PO$_4$)$_2$ is still not fully understood, it seems that the organic acids may contribute to its dissolution [3]. Organic acids in soils are the metabolic products of plants released by plant roots and soil microorganisms [4]. Organic acids that responsibility to the P-minerals solubilization include oxalic, citric, lactic, gluconic, malic, tartaric, citric, succinic, lactic, formic and acetic acids [5]. Behera et al. stated that among the phosphate solubilizing microorganisms, bacteria are the most effective ones [6]. Some farmers of West Nusa Tenggara (NTB) Province Indonesia have mostly used phosphate biofertilizers (such as Biotara and Bio P-2000Z) to improve the solubilising phosphate minerals in soils. However, the bio-fertilizers are ineffective in playing its role, because the bacteria contained in the bio-fertilizers fail to adapt to the new soil environment, because the bacteria were isolated from different soil characteristics where the bacteria were isolated. Bacteria contained in the bio-fertilizers come from acid mineral soils which are rich of variscite (AlP$_2$O$_7$.2H$_2$O) and strengite (FePO$_4$.2H$_2$O) minerals [7], so that it is probably not suitable to be used in dry land with neutral pH, like dry land in NTB.

So the aim of this study is to obtain the combination of drought-tolerant phosphate solubilizing bacteria which has high potential in dissolving Ca$_3$(PO$_4$)$_2$. The drought-tolerant phosphate solubilizing bacteria will be able to be further use as source of isolates for phosphate-biofertilizers on dry land.

2. Materials and methods
To obtain the best combination of drought-tolerant phosphate solubilizing bacteria, there are three bacteria investigated in this study, namely: (1) Pseudomonas azotoformans (A), (2) Acinetobacter baumannii (B) and (3) Bacillus paramycoides (C). These three bacteria were combined into four combinations: (1) A and B bacteria, (2) A and C bacteria, (3) B and C bacteria, and (4) A B and C bacteria. Each treatment was replicated three times. These bacteria were previously isolated from the rhizosphere of plant species (Tithonia diversifolia) which grows and develops on the dryland of Lombok Island, Indonesia [8]. These bacteria are tolerant to osmotic pressure of 0.41 MPa, which created by adding 15% Poly Ethanol Glycol (PEG) 6000 in pikovskaya solid medium [9].

Firstly, to test the solubilizing potential of the bacteria, each of the combination of the bacteria was inoculated in pikovskaya solid medium for 10 days. The Pikovskaya solid medium used was consisted of glucose (10.0 g L$^{-1}$), (NH$_4$)$_2$SO$_4$ (0.5 g L$^{-1}$), NaCl (0.5 g L$^{-1}$), KCl (0.2 g L$^{-1}$), MgSO$_4$.7H$_2$O (0.1 g L$^{-1}$), MnSO$_4$. H$_2$O (0.001 g L$^{-1}$), yeast extract (0.5 g L$^{-1}$), Ca$_3$(PO$_4$)$_2$ (5.0 g L$^{-1}$), and plus agar (15.0 g L$^{-1}$) (Rao and Sinha, 1963). Every 2 days it was observed the diameter of the bacterial colony and the clear zone developed. The solubilizing potential of the bacteria was measured by phosphate solubilizing index (PSI) as follows [10]. The best potential ability of the bacteria in solubilizing phosphate is assessed from the highest PSI:

$$\text{PSI} = \frac{\text{diameter of the clear zone} - \text{diameter of the bacterial colony}}{\text{Diameter of the bacterial colony}}$$

The second method of assessing the potential solubilizing bacteria by inoculating each combination of the bacteria in a 20 ml of pikovskaya liquid medium (non-agar) at a 100 rpm of rotary shaker for 32 hours. Then, each 1 ml of the suspension was taken and put into 10 Erlenmeyer glasses containing 100 ml of Pikovskaya liquid medium for 10 days. Every 2 days, the concentration of phosphate was measured using Olsen method from 2 Erlenmeyer glasses, including the pH of the medium [11,12]. The highest P concentration shows strongest combination bacteria in dissolving inorganic phosphate.

3. Results and discussion
3.1. Phosphate solubilizing potential assessed from PSI
The PSI of each combination of the bacteria is presented in Figure 1. It can be seen that the ABC bacterial combination produced the highest PSI compared to other combinations in every 2 days of observation. PSI of all combinations tend to increase during the incubation period. The highest potential in dissolving
inorganic phosphate obtained by the ABC bacterial combination is probably due to the largest amount and variety of organic acids produced by the bacteria. Khan et al. explain several factors influencing the solubility of P are the amount and variety of organic acids [13]. The PSI obtained by the ABC bacterial combination reaches 2.55 on the 10 days of the incubation period. This PSI value is relatively similar to the PSI of *Flavobacterium* sp (2.57), but this is higher than the PSI of *Enterobacter* sp (1.29), in which both bacteria were isolated from peat soil [14].

**Figure 1.** PSI of all the bacterial combinations during the incubation period.

### 3.2. Soluble phosphate concentration in pikovskaya liquid medium

Figure 2 shows the solubility phosphate concentration in pikovskaya liquid medium inoculated by the four bacterial combinations. The phosphate dissolving ability of each bacterial combination in the pikovskaya liquid medium was different. The data in figure 2 show that on the first of two days of the incubation period, there was a drastic increase in the concentration of P-dissolved in the pikovskaya liquid medium of each bacterial combination, while the dissolved phosphate concentration continued to increase until the end of the incubation period. In the figure 2, it is also shown that a relatively high P-dissolved concentration occurs in the ABC bacterial combination during the incubation period. The P-dissolved concentration of the ABC combination reaches 0.29 % of the total (Ca₃PO₄)₂ contained in the pikovskaya liquid medium on the ten days of the incubation period. While the other combinations are below that value, 0.21% for the A and B combination, 0.19% for the B and C combination, and 0.18% for the A and C combination, respectively. These results indicate that the ABC bacterial combination has the most potential ability in dissolving (Ca₃PO₄)₂.

**Figure 2.** The concentration of P-dissolved (%) in the pikovskaya liquid medium of all the bacterial combinations during the incubation period.
3.3. **pH changing in pikovskaya liquid medium**

Figure 3 presents the changing pH in the pikovskaya liquid medium of all bacterial combinations during the incubation period. The data in figure 3 show a drastic decrease in pH values occurred on the first of 2 days of the incubation period, and then the pH of the medium is relatively constant.

![Figure 3. The change of pH in Pikovskaya liquid medium during incubation period.](image)

The pattern of the changing pH in this study is slightly different to the result of a previous research at which a drastic decrease in pH values took place up to four days then the value was constant [15]. A decrease in pH values indicates that in the pikovskaya liquid medium there was an accumulation of organic acids excreted by the bacterial combinations. Organic acids produced by the bacteria decrease the pH which is considered to be responsible for phosphate solubilization activity [16]. The result of this study shows that the lowest pH occurred in the pikovskaya liquid medium containing the ABC bacterial combination. They change the pH from 6.24 to 4.84 for 10 days incubation period. This pH reduction rate seems smaller than that of the pH of pikovskaya liquid medium which was inoculated by *Pseudomonas* sp TPSB24 (pH 7 to 4.02) in a 120 hour incubation period [17].

Subsequently, in Figure 4, it can be seen that the increased concentration of the P-dissolved in the pikovskaya liquid medium due to the ABC bacterial combination which is in line with the increase of the PSI values, with the correlation value of 0.89. This increased trend is similar to the previous studies, where the correlation of P-dissolved and PSI was 0.87 [18]. In contrast, Suliasih and Widawati [19] found low correlation between P-dissolved and PSI.

![Figure 4. The pattern of increasing P solubility and increasing PSI of the ABC bacterial combination.](image)

The relationship between the change of pH and the amount of P-dissolve in pikovskaya liquid medium is presented in Figure 5. The first two days of the incubation period, the pH of the medium drops 1.26 units from pH 6.24 to 4.98, meanwhile, the P-dissolve increases from 0 to 0.17% of the total Ca$_3$(PO$_4$)$_2$ contained in the pikovskaya liquid medium. Furthermore, after two days of the incubation period, the
pH of the pikovskaya liquid medium is relatively constant. However, the P-dissolve tends to increase. These results explain that the increase of P-dissolve decrease is not always affected by the decrease pH. Several of the previous researches found the drastic increase of P-dissolve occurred at the beginning of the incubation period and followed by the gradual increase [20,21].

![Figure 5. Relationship between pH and P-dissolved of pikovskaya liquid medium of the ABC bacterial combination.](image)

4. Conclusion
Combination of *Pseudomonas azotoformans*, *Acinetobacter baumannii* and *Bacillus paramycoides* (A B and C bacterial combination) solubilize the largest amount of inorganic phosphate [(Ca$_3$(PO$_4$)$_2$] compared to the other combinations *Pseudomonas azotoformans* and *Acinetobacter baumannii* (A and B), *Pseudomonas azotoformans*, and *Bacillus paramycoides* (A and C), as well as *Acinetobacter baumannii* and *Bacillus paramycoides* (B and C). This three bacteria combination resulted in the highest dissolved P with 0.29% potential dissolution of the total Ca$_3$(PO$_4$)$_2$ contained in the liquid medium, followed by the A and B combination (0.21%), the B and C combination (0.19%), and the A and C combination (0.18%), respectively. Because this study is a laboratory scaled research, it is needed further research in order to know the effectivity of the combination of this three bacteria in dissolving soil P of dry land.

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