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The Capacity of Soil Bacteria, *Bacillus sp*^{rif} and *Pseudomonas sp*^{rif}, in solubilizing Soil Phosphate and Potassium

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Abstract, Increasing availability of phosphate (P) and potassium (K) in the soil can be driven by microbial activities, which are accurately able to dissolve P and K, known as nutrients hard to dissolve. The objectives of this research were to study the solubilizing activity toward soil P and K by P- and K- solubilizing bacteria in a sterilized and non-sterilized condition of Oxisol and Inceptisol. Marking procedure was performed on P- and K- solubilizing bacteria to scrutinize their activities in the soil. Marking process was conducted by utilizing resistance toward rifampicin antibiotic concentration of 50 µg.ml^{-1}. The results of this study shown that the increasing availability of P was evident in both soil conditions (sterilized and non-sterilized). In Oxisol, P availability increased by 48.86%, while in Inceptisol it reached an increase of 187.77%, compared to the initial concentration. Likewise, K availability in Oxisol increased by 4.53 times, and it rose by 5.26 times in Inceptisol. The activities of P solubilizing bacteria, to be able to increase soil P availability, were also able to enhance soil's K content. Similarly, the K solubilizing bacteria were also capable of increasing P availability in both soils.

Keywords: Oxisol, Inceptisol, Phosphate – Potassium solubilizing bacteria

Introduction

Soil is a dynamic system with a great extent of microbial heterogeneity. Microorganism activity occurring in the soil exerts bearing impact on soil nutrient cycle. The availability of phosphorus and potassium nutrients is influenced by soil microbial activity in accelerating weathering and solubilization processes. Potassium is present in four forms in soil, which are K ions (K^+) in soil solution, as an exchangeable cation, tightly held on the surfaces of clay minerals and organic matter. Moreover, it is tightly held or fixed by weathered micaceous minerals and present in the lattice of certain K-containing primary minerals. Soil characteristics affecting the availability of K pertain to the number and type of clay minerals, cation exchange capacity, potassium buffer capacity, moisture, temperature, aeration, and soil pH. The inoculation involving bacteria, which can improve P and K availability in soils by producing organic acids and other chemicals, stimulates the growth and mineral uptake of plants [1].

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A research result [2] corroborates that yeast *Torulaspora globosa* dissolves 38% of total alkaline ultramafic rock powder for 15 days of incubation, while *Aspergillus niger* dissolves 62% -70% of total ultramafic alkaline mineral, after 35 days of incubation [3]. The double inoculation of PSB (phosphate-solubilising bacteria), *Bacillus megaterium*, and KSB (potassium-solubilising bacteria) *Bacillus mucilaginosus* combined with P and K bearing mineral, significantly increases the availability of P and K in the soil, marked by an increase of approximately 25% for P and 15% for K, compared to the control [4]. Another research finding [5] evinces 5 KSBs (potassium-solubilizing bacteria) that produces organic acids such as Citric, Oxalic, Malic, Succinic, and Tartaric acid. A qualitative test in the study indicated a solubilization index ranging from 1.04 to 1.66. In the previous research [6] proved the ability of KSB to produce organic acids, among other things, citric acid, ferulate, malate, and coumaric, capable of accelerating K release from feldspar, leucite, and biotite. Besides, some KSBs produce enzymes, including Amylase, Protease, Lipase, Catalase, and Glucose.

Antibiotics resistance of KSB works against such antibiotics as Ampicillin, Tetracyclin, Amoxycillin, and Novobiocin. Antibiotic resistance has provided a potentially simple and effective method to genetically mark bacterial strains for monitoring after the introduction into complex ecosystems. The markers of microorganisms with antibiotic resistance are generally preferred methods for studying the activity of soil microorganisms. For example, Rifampicin resistance is the most commonly used marker to study population dynamics and survival of plant growth-promoting after their introduction in the rhizosphere [7] and disease-suppressing *Rhizoctonia solani* by *Pseudomonas Putida* strain $2C8^{rif}$ [8]. Investigated in previous studies, markers given to microorganisms through the exposure to an antibiotic such as rifampicin did not affect their activity on rhizosphere. One of the previous studies [9] has confirmed the ability of mutant *Pseudomonas putida* bacteria with rifampicin antibiotic, which is potent in colonizing roots in non-sterilized conditions.

This study aimed to learn the solubility capability of phosphate-solubilizing bacteria and potassium-solubilizing bacteria (*Bacillus* sp^{rif} and *Pseudomonas* sp^{rif}) in increasing the availability of P and K nutrients in Inceptisol and Oxisol, within sterilized and non-sterilized condition.

Materials and Research Method

1.1 Isolate Marker

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Testing the viability and activity of isolates *Bacillus sp* as phosphate-solubilizing bacteria and *Pseudomonas sp* as potassium-solubilizing bacteria under non-sterile conditions was marked by using antibiotic resistance Kirby-Bauer method. The administration of markers with the resistance test against Rifampicin antibiotic up to a concentration level of 50 $\mu g.mL^{-1}$ was carried out gradually. Both isolates that have been characterized are identified as *Bacillus sp*^{rif} and *Pseudomonas sp*^{rif}

1.2 Solubility Test in Solid Selective Media by Marked Isolate

The test in this regard scrutinized the solubility of Bacillus sprif and Pseudomonas sprif, and It was carried out in Alexandrov and Pikovskaya solid medium by calculating the solubility index (SI) using the following formula on seventh days:

$$SI = \frac{\text{Clear zone diameter}}{\text{Colony diameter}}$$

1.3 The Examination on Soil's phosphate and potassium Solubility

The solubilization activity test was performed on two soils with different characteristics (Table 1) and within two different conditions (sterilized and non-sterilized). A completely randomized design was made operative, which involved soil factors including sterilized Oxisol (OS), non-sterilized Oxisol (ON), sterilized Inceptisol (IS) and non-sterilized inceptisol (IN). The second factor was concerned with the isolates: control group (B0); PSB (*Bacillus sp*^{rif}) and KSB (*Pseudomonas sp*^{rif}). The study operationalized 12 treatment combinations with three replications. 200 g soil was inoculated with each bacteria at a cell density of 10^7 per gram soil. The soil condition was maintained to resemble field capacity condition and incubated for a month. Soil sterilization is prepared two times with autoclave at 121^0C for 15-20 minutes at 1-atmosphere pressure. Regularly, particularly on the tenth day, twentieth day, and thirtieth day, soil pH, phosphate concentration (Bray extract), and concentration of potassium (Citric acid extract) were analyzed.

Table 1. The Soil Characteristics

Type of Analysis	Unit	Value	
		Inceptisol	Oxisol
pH H_2O (1:2,5)		5,67	4,54
pH KCl (1:2,5)		4,78	3,85
Sand	%	21.44	41
Silt	%	29.81	26

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Clay	%	48.76	33
Texture		Clay	Clay loam
K-available (Amonium acetate 1M pH 7)	(cmol(+)/kg)	0,08	0,07
P_2O_5 (Bray)	mg.kg^{-1}	23,00	7,10
P_2O_5 (HCl 25%)	mg.100g^{-1}	121,50	20,10
C-Organic	%	1,21	2,18

3. Research Results

3.1 Antibiotica Resistance

The examination isolates resistance against Rifampicin antibiotic proved that both bacteria were quite resistant at a concentration of 50 µg.mL^{-1}. The use of rifampicin antibiotic was quite useful as a marker in bacteria tested in various soil conditions. Previous research [9] corroborated that, for *Pseudomonas* ecology test in the field, Rifampicin antibiotic resistance marker was applied to *Pseudomonas putida* strain WCS358 to a concentration of 250 µg.ml^{-1} and it concluded that rifampicin was stable to be applied as a marker for *Pseudomonas putida* strain WCS358 in potato rhizosphere. Other studies [10; 8] also have concluded that the use of rifampicin in *Pseudomonas fluorescens* and *Pseudomonas putida* is useful material for environmental testing of mutants resulting from there. Continuous exposure to rifampicin antibiotic resulted in resistance arising from the mutations of β-RNA polymerase subunit gene and RNA polymerase, which changed due to usually functional mutation resistant to rifampicin-triggered inhibition.

3.2 Solubility Index

The activity of the two mutant bacteria in the solubilization of P and K in vitro setting within both Pikovskaya and Alexandrov's media was still detected. The excellent solubilizing capability was shown by two mutant bacteria, namely *Bacillus sp^{rif}* and *Pseudomonas sp^{rif}*. Solubilization by *Bacillus sp^{rif}* has a solubility index (SI) of 2.5 with the widest clear zone diameter of 0.75 cm on Pikovskaya media, while *Pseudomonas sp^{rif}* displayed an SI value of 3.3 with maximum “clear zone” diameter of 1 cm on Alexandrov's media. The solubility index evinced the bacteria's ability to dissolve the sources of P and K present in the media. Based on the criteria applied by the researchers on the results of previous studies [6], both bacteria possessed intermediate-degree solubilizing capabilities (SI ≤ 2.00 ≤ 4.00), but their rates of solubilization were dissimilar in that PSB (*Bacillus sp^{rif}*) was found at fast solubilizing category

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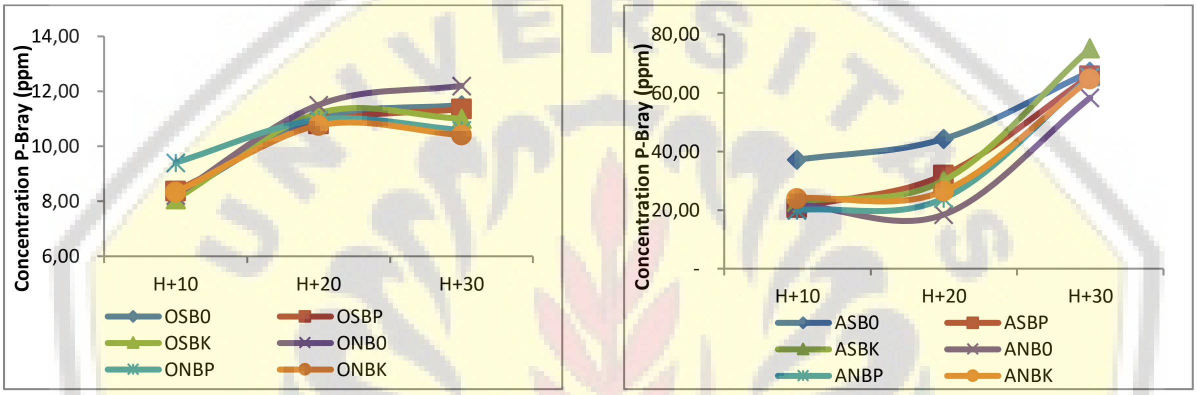
(<3 days) whereas KSB (*Pseudomonas sp*^{rif}) was proven to be at slow solubilizing category (5 days).

3.3 The Test on Soil's P Solubility

Oxisol and Inceptisol tested have low P and K concentrations, acid conditions with low organic content (Table 1). The soil conditions affect the activity of the isolates because, for activities, it takes nutrients and organic carbon. The test results concerned with soil solubility activity showed an increase in all treatments up to the 30th day in Oxisol and Inceptisol soils (figure 1).



(a) (b)

Figure 1. The Concentration of P-Available in Oxisol (a) and Inceptisol (b)

The presence of microbial activity of both PSB (*Bacillus sp*^{rif}) and KSB (*Pseudomonas sp*^{rif}) in Oxisol soil caused P concentration to be lower than that of the control treatment with no microbial activity, although at the end of incubation the P concentration was still found higher than was the initial concentration (Figure 1). The high ratio of organic carbon to the availability of P in the soil led to the immobilization of P. Under the condition characterized by C : P ratio < 200, the mineralization of P started to occur [11], while the initial data showed C: P ratio available > 200. This condition resulted in a larger immobilization net and showed P in the soil solution used by the existing microbes, thereby declining the P concentration in the soil solution. Oxisol constitutes highly weathered soil, so the phosphate is bound to Al, Fe or occluded-Fe, making it unavailable to plant.

The pattern of P solubilization between Oxisol and Inceptisol was different, although the concentration at the end of incubation in both soils increased, compared to the initial soil concentrations (Figures 1a and 1b). In Inceptisol, a substantial increase was evident after the

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20th day. The dissolved phosphate in Inceptisol soils was higher than that of Oxisol. Besides, P immobilization through the use of phosphate by microbes still maintained the concentration of P available in the soil solution. In Inceptisol, C: P ratio was lower than that in Oxisol. The lower the C: P ratio was, the more likely mineralization of P was to occur, and the lower net immobilization would become.

The sterilized and non-sterilized soil conditions did not make a significant difference in the solubilization of P due to the microbial activity applied. *Bacillus sp*^{rif} and *Pseudomonas sp*^{rif} were able to perform well in any soil conditions. As a corollary, the ability to compete against indigenous microbes was quite sound. Based on table 2, *Pseudomonas sp*^{rif} also possessed the ability to solubilize P from both Oxisol and Inceptisol.

In their activity, bacteria secreted several metabolites, including organic acids. The results of a previous study [6] point out that KSB produces organic acids such as Citric, Ferulic, and Coumaric. In the same vein, another study [5] corroborates that KSB produces Citric, Oxalic, Malic, Succinic, and Tartaric acid. Furthermore, PSB also produces organic acids in its activities, encompassing Citric, Oxalic, Malic, Succinic [12], Citrate, Oxalate, Succinate, Fumarate, Acetate, Propionate, and Butyrate [13]. One of the mechanisms of solubilizing P, mainly resulting from the presence of organic anions, is done through several mechanisms, inter alia (1) organic anions competing against orthophosphates ion on the surface of the positively-charged colloidal site; (2) the release of orthophosphate ion from metal-P bond through the formation of organic metal complexes [14]; and (3) the modification of surface charge of the site by organic ligands [15].

Table 2. The Concentration of P-Available at the End of Incubation (ppm)

Innoculant	Oxisol		Inceptisol	
	sterilized	non-sterilized	sterilized	non-sterilized
PSB (*Bacillus sp*^{rif})	11,36	10,59	67,15	69,17
KSB (*Pseudomonas sp*^{rif})	11,01	10,41	75,25	69,28

According to [14] the presence of certain organic anions derived from the decomposition of organic matter, microbial activity, or root secretion may affect the absorption of P through the competition of surface site or lower the adhesion site through solubilization. Besides, organic acids with low molecular weight lowered P by Al oxide, Fe, or allophane clay minerals due to their high affinity [16]. The addition of organic acids with low molecular weight also effectively triggered Al and Fe from Fe-P and Al-P, allowing the solubilization of P to occur

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[17]. Organic ligands such as tartaric, oxalate, malate, and citrate containing carboxyl (COOH), aliphatic-OH, phenolic-hydroxyl groups were proven highly effective in mineral solubilization and chelate formation enriched with such elements as Al, Fe, Ca, and other elements, and declining pH level of media [18].

The reaction of phosphate solubilisation by organic acids is formulated as following.

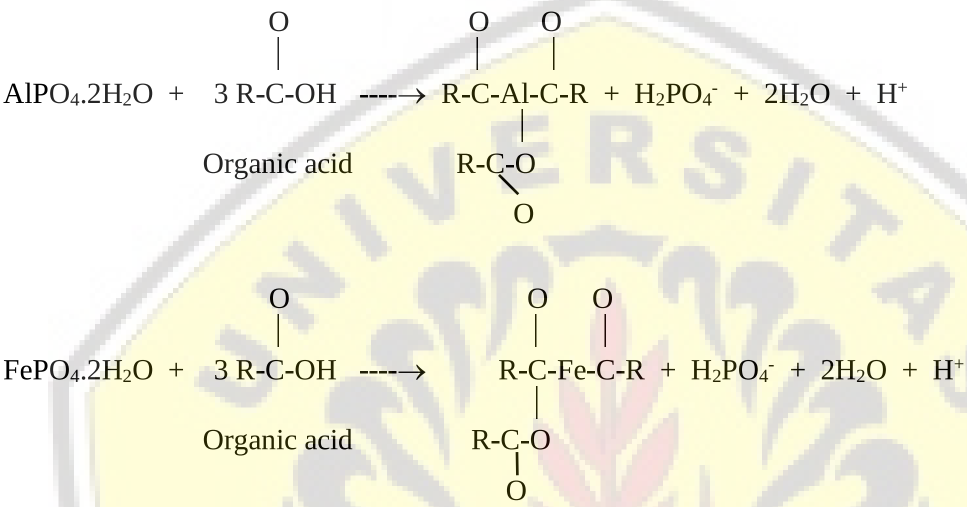

Table 3. Phosphate Released from soils by Inoculant after 30 Days of Incubation ($mg.kg^{-1}$)

Treatments	Oxisol (sterilized)	Oxisol (non-sterilized)	Inceptisol (sterilized)	Inceptisol (non-sterilized)
Control	4.00±0.65	4,69±0.57	44.29±3.82	40.82±10,00
PSB (*Bacillus sp^{rif}*)	3.85±0,46	3.08±0,34	44.15±2.28	46.17±5.49
KSB (*Pseudomonas sp^{rif}*)	3,50±0,22	2.90±0,58	52.25±4.82	46.28±6.35

During the 30-day incubation, the average of P concentration in Inceptisol increased considerably to a maximum extent of 187.77%, while in Oxisol similar increment peaked to a maximum extent of 48.86%. The activity of inoculant, especially *Bacillus sp^{rif}* release phosphate at Oxisol maximum of 4.31 $mg.kg^{-1}$ while at Inceptisol 51.66 $mg.kg^{-1}$ (table 3). Oxisol was highly weathered soil, making possible P to precipitated with Al or Fe. Besides, P also bound with Fe and Al oxides, as well as in the form of occluded P. In Inceptisol, some adsorption complexes were predominantly Ca and Fe ions, so P bound to Ca and or Fe. Beside P-organic form, P-inorganic form in soil consists of several fractions. The proportion or percentage of each fraction influenced by several factors, including the type of soil. The distribution of inorganic P fraction (%) in some soil types in Malaysia and Indonesia [19] shows the fractionation of P in Oxisol and Inceptisol soils as follows:

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Table 4. The Fraction Distribution of Inorganic P (%)

Soil Order	The Distribution of P-inorganic (%)					
	Soluble P	Ca-P	Fe-P	Al-P	Occ Fe-P	Occ Al-P
Oxisol	0,15	3,9	35,6	4,5	50,8	4,9
Inceptisol	0,9	21,3	38,9	5,6	39,4	6,1

Soil dominant form P bound with Occluded Fe (OP) is more difficult to dissolve (Table 4). The results [20] show that the OP is not dissolved with the addition of soil amendment, so the concentration is still high, while the other P form increases its solubility. The fractionation of P in Oxisol of West Java was obtained by the sequence of the greatest as follows: reductan Fe-P> Fe-P> Occluded-P> Ca-P> Al-P> soluble-P [13], therefore release of P on Oxisol is lower than Inceptisol.

The soil acidity indicated an increase at all treatments until the end of incubation in both Oxisol and Inceptisol (see Figure. 2). Phosphate-solubilizing bacteria and potassium-solubilizing bacteria generally produced organic acid. However, the presence of organic acid did not diminish the pH level of both soil types. The carboxyl groups from organic acid developed negative charge as the positively charged H was removed. When the pH of soil increased, the release of H from carboxyl groups aided in sustaining the increase of pH and at the same time created the CEC (negative charge). The presence of negative charge on the surface of the clay mineral would attract Ca, Fe or Al cations, consequently increasing phosphate availability.

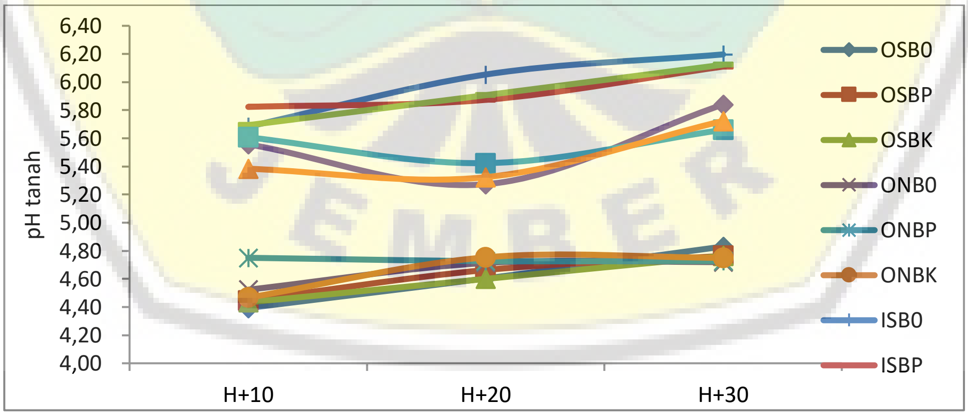

Figure 2. The Change of Soil pH in Oxisol and Inceptisol

3.4 Potassium Solubilisation

The concentration of K-soluble citric acid in two soil types increased along with increasing period of incubation in the same pattern (Figure 3). The sterilized and non-sterilized soil

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conditions also did not affect the activity of the two solubilizing bacteria in both soil types, resulting in increased concentration of K. *Bacillus sp^{rif}* and *Pseudomonas sp^{rif}*, both of which are mutant bacteria against rifampicin resistance. The enhanced concentration, however, did not affect the capacity of bacteria as K-solubilising agent. Findings of research [21] using antibiotics resistant against Mutant potassium-releasing bacterial strain of *Bacillus edaphicus NBT* with rifampicin antibiotic (150 mg.l^{-1}) also reveal excellent activity in increasing P and K concentrations in soil, so that P and K uptake by cotton plants and Rape is greater than those without inoculation. Re-isolation at the end of incubation performed on non-sterilized soil conditions showed the presence of both mutant bacteria. In the 5th week, the mutant potassium-releasing bacterial strain of *Bacillus edaphicus NBT* was still established in cotton and rape rhizosphere. One of the mechanisms for solubilizing the soil potassium by the activity of the microorganisms resulted from excreted organic acids, therefore triggering the organic cation to be stimulated by Si ions and releasing K in the solutions.

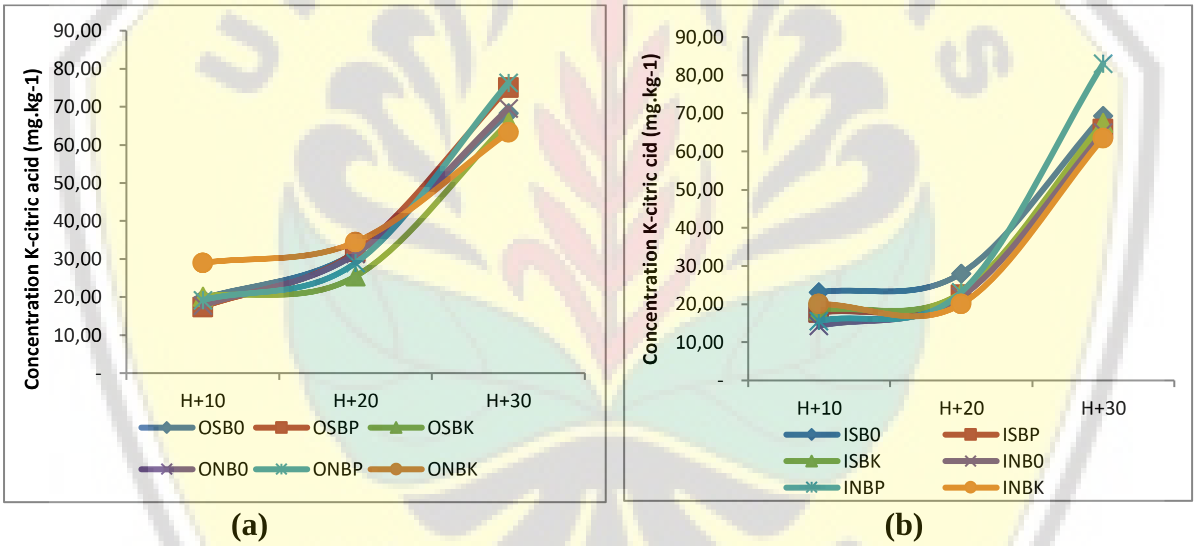

Figure 3. The Concentration of Potassium Solubilisation (Citrate Acid extract) in Oxisol (a) and Inceptisol (b)

The concentration of potassium in Oxisol increased by 3.78 to 4.53 times greater than its initial concentration, whereas Inceptisol rose by 4.32 to 5.26. The increase in Inceptisol was more intense than that in Oxisol because Inceptisol generally was dominated by smectite clay minerals and some kaolinite, and it was derived from clay sediment primary material [22]. The concentration of soil K was dependent on the amount of smectite clay minerals in the soil. Clay mineral Smectite could fixate K at that mineral layer interspace where the fixated K was reserved exch-K for plants through release and desorption processes, resulting in the higher release of K in Inceptisol. The availability of soil K relied on the process and dynamic of K in soil, especially the sorption and desorption process. Sorption and desorption of K in soil were

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determined mainly by the type and amount of clay minerals. Clay mineral type 2:1 adsorbed K and released K more intensely than did other clay minerals, such as type 2: 1: 1, 1: 1, oxide, and allophane [22]

In general, mineral composition and chemical properties of red soil include the presence of Oxisol particularly in the humid tropical area, characterized by sand mineral dominated by quartz and opaque, while clay mineral is dominated by kaolinite with additional mineral gibbsite, goethite, and hematite [23]. By contrast, according to another research [24], the clay fraction of Oxisols is dominated by (a) 1: 1 phyllosilicate; (b) oxides of Fe and/or Al, or the mixtures of (a) and (b). Clay minerals in soils of tropical climates such as Oxisols and Ultisols are dominated by kaolinite and halloysite, in addition to gibbsite and sesquioxides. The soil with a dominance of kaolinite clay and halloysite is characterized by the presence or position of K ion, which is not on the interspace layer because there is only one octahedral layer and one tetrahedral layer. Consequently, K fixated as a K-reserve, which can release in solution is also low (Figure 4).

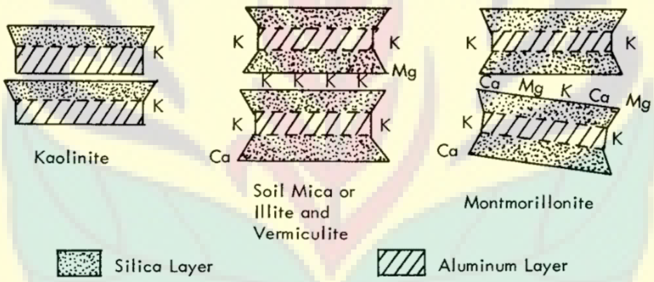

Figure 4. The Illustration of clay Mineral Structure (type 1:1), Illite (type 2:1 non exchangeable), and montmorillonit (type 2:1 exchangeable)

Both *Bacillus sp*^{rif} and *Pseudomonas sp*^{rif} were able to solubilize phosphate and potassium in both soils, although each had distinctive solubility capability, it is line with the study [25] which proves the ability of *Bacillus mucilaginosus* in increasing P and K availability. The same finding is obtained in another research [4] using phosphate solubilizing bacteria, (PSB) *Bacillus megaterium var. Phosphaticum* and potassium solubilizing bacteria (KSB) *Bacillus mucilaginosus*. The mechanism of solubilizing phosphate and soil potassium was relatively similar in that it was carried out through acidification, chelation, and exchange reactions [26], as well as through the production and excretion of organic acids.

4. Conclusion

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Both isolates of PSB (*Bacillus sp*^{rif}) and KSB (*Pseudomonas sp*^{rif}) have been found effective to solubilise the phosphate and potassium of Oxisol and Inceptisol soils in both sterilized and non-sterilized conditions. Phosphate or potassium solubilizing in Inceptisol soil has been proven greater than that in Oxisol. The sterilized and non-sterilized soil conditions have revealed no bearing impact on the solubilizing activity in both isolates.

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