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Research Article
Isolation and Solubilisation of Inorganic Phosphate by *Burkholderia* spp. from the Rhizosphere of Oil Palm

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Abstract

Background and Objective: Phosphate-solubilising bacteria (PSB) are useful for plant growth. They inhabit different soil ecosystems such as colonizing the root environment. The aims of this study was to isolate PSB from oil palm rhizosphere and to conduct a comparative analysis of the solubility of inorganic phosphates. **Materials and Methods:** Rhizospheric soil samples at 0-20 cm depth collected from the distance of 2 m away from the palm were isolated and their chemical and physical properties were analyzed. Qualitative estimation of the suspected PSB was screened by inoculating and growing them at 27°C for 10 days on NBRIP agar medium with bromophenol blue. Their abilities to solubilize $AlPO_4$, $FePO_4$ and $Ca_3(PO_4)_2$ were examined. Phosphate solubilizing activities were tested on the NBRIP growth medium by analyzing solubilisation efficiency and soluble-P content. Genomic DNA was isolated using QIAamp® genomic DNA kit. **Results:** A total of 15 PSB were successfully isolated from oil palm rhizosphere. During 5 days of incubation, isolate K3.1, A4 and K3.3 solubilized 53.5, 63.5 and 58.6 mg L^{-1} phosphate inoculated in Al_3PO_4, Fe_3PO_4 and $Ca_3(PO_4)_2$, respectively. Based on the 16S rRNA gene sequence analysis, those isolates were closely related to *Burkholderia arboris*, *Burkholderia gladioli* and *Burkholderia seminalis*, respectively. In soil analysis, P_2O_5, C-organic and CEC had positive correlation with the total PSB. **Conclusion:** The existence of P promoting bacteria in oil palm rhizosphere may offer effective solution on biofertilizer agent for sustainable agriculture.

Key words: *Burkholderia arboris*, *Burkholderia gladioli*, *Burkholderia seminalis*, oil palm, P solubilising bacteria, rhizosphere, inorganic phosphates

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Phosphate (P) is the main macronutrient compound which is essential for plant growth. The maximum part of soil phosphate, approximately 95-99% is present in the form of insoluble phosphates and hence it cannot be easily utilized by the plants[1]. Depending on some environmental and biological factors, it can be the main growth-limiting nutrient[2].

Phosphate is most readily available[3,4] at pH around 6-7. The insoluble forms of P such as tricalcium phosphate ($Ca_3(PO_4)_2$), aluminum phosphate ($AlPO_4$) and iron phosphate ($FePO_4$) may be converted to soluble P by phosphate-solubilising bacteria (PSB) inhabiting different soil ecosystems[5,6,7]. The role of bacteria in dissolving P is more widely used because bacteria are also able to produce other acids such as amino acids, vitamins and growth-promoting hormones such as gibberellins and IAA which are useful for plant growth[5]. PSB can play an important role in dissolving both of fertilizer P and bound P in the soil that is environmentally friendly and sustainable[8]. Moreover, according to Behera *et al.*[9], PSB in solubilizing P derived from soil or fertilizing activities in several agricultural fields depend much on their abilities to secrete organic acids such as citric acid, formic acid, oxalic acid, lactic acid, acetic acid and acid malat.

Despite the agronomic benefits that may be provided by PSB, their abundance in soil is not always sufficient to compete with other microorganisms established in the rhizosphere[10]. In order to establish sustainable oil palm plantation especially on marginal soils and those with less dependent on inorganic fertilizers, it needs to identify and search the important soil bacteria which have high influence on plant growth, such as PSB[11]. The aims of this study were to isolate PSB from oil palm rhizosphere and to conduct a comparative analysis of the solubility inorganic phosphates from identified PSB.

MATERIALS AND METHODS

Time and venue: The study was performed in oil palm cultivated area belonged to PT Sampoerna Agro Tbk. located in Ogan Komering Ilir, South Sumatra Province, Indonesia (3°47′ 04.1″ S to 3°47′12.8″S and 105°10′26.6″E to 105°10′ 59.0″E). It lasted from May to October, 2016. The palm was at productive stage and aged about 10 years old after planting. The soil was classified as acidic soil.

Soil sampling and bacterial isolation: Rhizospheric soils at 0-20 cm depth were collected from 3 different points at the distance of 2 m from each palm. A total of 6 palms were used in this study. Those collected soil samples were then mixed and composited into one samples with total fresh weight of 3 kg. About 1.5 kg of soil was used for the analysis of chemical (soil pH, N-total, C-organic, CEC, Cation exchange of K, Ca and Mg, P-total, available P and Exch. Al and H) and physical (composition of silt, clay and sand) properties. The rest was used for bacterial isolation.

Ten gram of fresh rhizospheric soil samples were transferred into 250 mL Erlenmeyer flasks containing sterilized 90 mL of 0.85% NaCl solution. A series of ten-fold dilutions of the suspension was made by pipetting 1 mL aliquots into sterilized 9 mL of 0.85% NaCl solution. Aliquots of 0.1 mL of the sample from each of these dilutions were spread on to a petri dish with National Botanical Research Institute Phosphorus (NBRIP) agar medium containing 10 g of glucose, 3 g of $AlPO_4$ or 5 g of $FePO_4$, 5 g of $MgCl_2.6H_2O$, 0.25 g of $MgSO_4.7H_2O$, 0.2 g of KCl, 0.1 g of $(NH_4)_2SO_4$ and 10 g of gellan gum agar in 1 L distilled water[12]. The pH of the media was adjusted to 5.0 using HCl. The plates were incubated for 7 days in an incubator at 30°C. The colonies with halo zone were considered to be PSB. These bacteria were further purified by re-streaking on the fresh NBRIP agar plates at 30°C.

Qualitative and quantitative estimation of phosphate solubilisation: An efficient protocol was developed for qualitative screening PSB, based upon visual observation. Qualitative estimation of all the suspected PSB was screened by inoculating and growing them at 27°C for 10 days on NBRIP agar medium with bromophenol blue. The diameter of the halo zone was measured after 42 h, up to 10 days. The phosphate solubilisation efficiency (PSE) was identified by measuring the total halo zone of the colony and the colony diameter[13]. The calculation was as follow:

$$PSE = \frac{(\text{Colony diameter+Halo zone diameter})}{\text{Colony diameter}}$$

The quantitative bioassay was carried out using erlenmeyer flasks (250 mL) containing 100 mL NBRIP agar media inoculated using bacterial isolates with approximately 1×10^8-10^9 Colony Forming Units (CFU) per mL following Saraswati and Sumarno's method[14]. The bacteria were inoculated in the medium having pH 5.0, which was adjusted before autoclaving the medium. The flasks were incubated at

30°C in a shaker for 5 days at 100 rpm. The cultures were collected for centrifugation for 10 min at 5,500 rpm. The supernatant was decanted and filtered through Whatman No. 41 filter paper. The available P content in the supernatant was estimated by the phosphomolybdate blue complex colorimetric method at 660 nm wavelength[15]. From this method, the abilities of selected isolates to solubilize $AlPO_4$, $FePO_4$ and $Ca_3(PO_4)_2$ were examined.

DNA extraction, PCR amplification and sequencing of 16S rRNA gene: Genomic DNA was isolated using QIAamp® genomic DNA kit following the manufacturer's instructions and 16S rRNA gene was amplified using the universal primers, 27f (5'AGAGTTTGATCCTGGCTCAG 3') and 1492r (5' TACGGCTACCTTGTTACGACTT 3'). The PCR thermal cycling conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of denaturation (1 min at 94°C), annealing for 1 min at 57°C and extension for 2 min at 72°C, followed by a final extension at 72°C for 8 min.

PCR amplified products were separated on 1.0% agarose gels in 1×TBE buffer at 70 V cm^{-1} for 20 min. Partial 16S rRNA genes of selected isolates in each group were sequenced by FIRSTBASE, Singapore and compared to that of other bacteria by way BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). In the best isolate(s) (phosphate-solubilizing ability) and three isolates were selected to sequence and the results were compared to sequences of GenBank based on partial 16S rRNA sequences to show relationships between PSB strains and a phylogenetic tree was constructed by the maximum-likelihood method using the MEGA software ver. 6.06 based on 1,000 bootstraps.

Statistical analysis: The *in vitro* experiment was conducted in complete randomized design with 3 replications and data were expressed as the mean value±standard error (SE). The treatments of identified isolates and inorganic phosphate solubilisation were used. The treatment of inorganic phosphates solubilisation consisted of $AlPO_4$, $Ca_3(PO_4)_2$ and $FePO_4$. All data were analyzed using the Predictive Analytics Software (PSAW)'s statistical software and mean differences were separated using Duncan's multiple range test at the 5% level of probability.

RESULTS AND DISCUSSION

Soil analysis: The soil collected from this study contained majority 75.27% sand, minor proportion of silt (4.21%) and the clay was 20.53% (Table 1). According to Hardjowigeno[16], it was classified as acid with soil pH at 4.75. It contained low N, medium C-organic, low CEC and very low exchangeable K, Ca and Mg as well as available P and total P. The exchangeable Al and H were very low.

Table 1: Soil characteristics collected from the rhizosphere (0-20 cm layer) of oil palm

Soil characteristics	Value	Classification
Sand (%)	75.27±2.46	-
Silt (%)	4.21±1.09	-
Clay (%)	20.53±1.74	-
pH H_2O	4.75±0.09	Acid †
N-total (%)	0.19±0.08	Low †
C-organic (%)	2.14±0.22	Medium †
CEC (cmol (+) kg^{-1})	9.34±2.33	Low †
K (me/100 g)	0.09±0.01	Very low †
Ca (me/100 g)	0.22±0.08	Very low †
Mg (me/100 g)	0.06±0.02	Very low †
P_2O_5 (ppm)	18.07±2.57	Very low †
Available P bray II (ppm)	3.47±2.06	Very low †
Exchangeable Al (%)	1.09±0.20	Very low †
Exchangeable H (%)	2.71±0.41	Very low †
PSB (log CFU g^{-1} dry soil)	5.01±0.30	

†: Classification of the ranges based on Hardjowigeno[16]

According to Sanchez[17] and Rao *et al.*[18], the natural acidification process in acidic soils had caused Al toxicity, deficiencies in Ca, Mg and Mo and a frequently low availability of P. Moreover, they found that in acid mineral soils, K and N tend to be deficient because they are usually highly weathered or have low organic matter content. The severe limitation of inorganic phosphate in acid soils was also reported by Zheng *et al.*[4]. Most of the soil analysis results reported in this study had agreed with those findings.

Total PSB reported in the present study was 5.01 or 2.24×10^5 CFU g^{-1} of soil. It was higher than those reported by several studies in acidic soil in Kenya, Uruguay and Argentina. Ndung'u-Magiroi *et al.*[19] reported the amount of PSB in 13 different sites of Kenya ranging from 0.38 to 9.1×10^5 CFU g^{-1} of soil. Similarly, Azziz *et al.*[2] reported that PSB present under crop-pasture rotations in Uruguay varied from 0.65 to 62×10^5 CFU g^{-1} of soil. In contrast, Fernandez *et al.*[20] recorded a low amount of PSB, located between 0.03 and 0.08×10^5 CFU g^{-1} of soil, in the most productive region of the Argentina.

Phosphate solubilisation efficiency (PSE): The isolated bacterial strains were capable of solubilizing $Ca_3(PO_4)_2$ in NBRIP medium and they formed large halos with varied intensity (Fig. 1) and the halo-zone increased with increase of colony diameter. Phosphate levels during 10 days of incubation showed high phosphate solubilization activity. These findings were similar as reported by Li *et al.*[21].

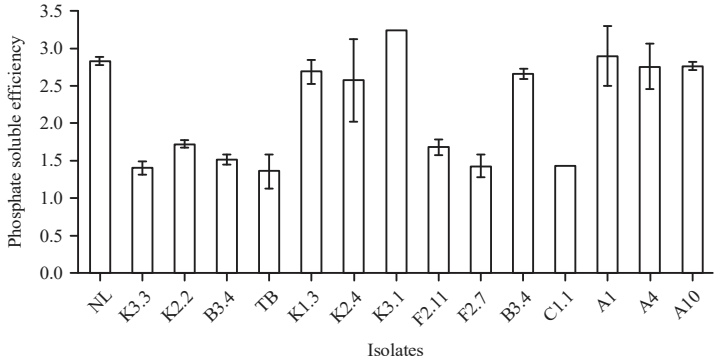

Fig. 1: Phosphate soluble efficiency index after 10 days of incubation period

Table 2: Iron, aluminum and calcium phosphates solubilisation by the selected isolates

	Soluble phosphate (mg L^{-1})		
Isolates	Strengite-iron phosphate	Variscite-aluminium phosphate	Apatite-calcium phosphate
A1	5.5±0.25^{a}	1.7±0.44^{a}	6.3±0.56^{a}
A10	7.3±0.19^{b}	45.0±1.98^{i}	51.7±0.38^{h}
A4	63.5±2.28^{h}	4.6±0.18^{b}	57.7±1.59^{kl}
B3.10	49.1±1.71^{e}	7.6±0.14^{d}	56.4±0.55^{jk}
B3.4	4.1±0.03^{a}	22.7±1.25^{e}	26.7±0.79^{d}
C1.1	4.3±0.05^{a}	43.1±0.61^{h}	47.6±0.47^{g}
F2.11	22.1±0.56^{c}	0.7±0.36^{a}	22.1±0.24^{c}
F2.7	4.3±0.27^{a}	25.3±0.86^{f}	29.6±1.09^{e}
K1.3	4.2±0.29^{a}	48.7±1.29^{j}	54.6±0.89^{i}
K2.2	58.8±1.13^{g}	4.9±0.01^{b}	55.3±0.90^{ij}
K2.4	4.2±0.16^{a}	44.2±2.11^{hi}	47.7±0.81^{g}
K3.1	4.2±0.13^{a}	53.5±1.21^{k}	57.1±0.75^{k}
K3.3	53.6±1.99^{f}	6.3±0.13^{bc}	58.6±0.73^{l}
NL	4.6±0.13^{a}	36.1±1.52^{g}	39.3±0.35^{f}
TB	30.2±0.89^{d}	15.7±0.14^{d}	13.1±0.77^{b}

Values are given as Mean±SD for triplicate samples, means followed by the same letter (s) in each column are not significantly different at $p \leq 0.05$ by Duncan's multiple ranged test

Among the isolates, the highest solubilisation index was found on isolate K3.1 (3.2) followed by isolate A1 (2.9). The incubation period required for exhibiting the solubilisation index for both isolates was 5 days. For isolates A4 and A10, the solubilisation index was recorded at 8 days of the incubation. Lower solubilisation index was found on K2.4 (2.6) at the same incubation period. These solubilisation activities reported in the present study were similar with those in costal soils with the PSE value[13] of 2.63. In addition, Ktt.B2 isolated from tropical acidic soil solubilized phosphate[22] at 3.47.

Inorganic phosphate solubilisation: Among 15 collected isolates shown in Table 2, isolates A4, K3.1 and K3.3 were the most efficient in solubilizing $FePO_4$ at 63.5 mg L^{-1}, $AlPO_4$ at 53.5 mg L^{-1} and $Ca_3(PO_4)_2$ at 58.6 mg L^{-1}, respectively. At the most cases, all isolates were able to solubilize $Ca_3(PO_4)_2$ to a greater extent than $AlPO_4$ and $FePO_4$. Isolate K3.1 was able to solubilize $AlPO_4$ at 53.5 mg L^{-1} which was five times greater than isolate N1 from oxisol (9.32 mg L^{-1}) as reported by Prijambada *et al.*[23]. Moreover, the solubilisation of iron phosphate by isolate A.4 was similar to isolate M150 (61.3 mg L^{-1}) reported by Panda *et al.*[6] in the rhizosphere of maize. The huge variation in iron, aluminum and calcium phosphates solubilisation by the selected isolates might have indicated that the use of microbial phosphate solvent still faced several obstacles such as soil factor, since every soil type possessed different phosphate form.

Correlation between total PSB and soil physicochemical properties: The present study found that the total PSB was greatly affected by several soil physicochemical properties (Table 3). Those were with C-organic (r = 0.94), P_2O_5 (r = 0.85) and CEC (r = 0.84). Other physicochemical properties such as soil physics, pH, available P and macro- and semi macronutrients were not correlated. These findings was very similar to those reported by Vikram *et al.*[24] who found strong positive correlation between organic carbon ($r = 0.40$, $p<0.01$), available N ($r = 0.40$, $p<0.05$) and PSB population. However, pH and available P showed no significant correlation. A study performed by Mahanta *et al.*[25] reported that CEC was in strong correlation with P inflow rate and the requirement of internal P influenced PSB activity. The present study showed that available P was positively correlated with CEC (r = 0.72).

The unclear correlation of other chemical properties with the total PSB might have been explained that the abundance of PSB in the soil depended on plant species, soil microbial composition and soil condition[26]. Nahas[27] reported that diversity and density of the population of PSB and their bio-activity varied from soil to another according to their nutritional status [C, N and P] and their own effectiveness in phosphate solubilization. Next to N and K, phosphorus was an essential element of energy metabolism of all life forms.

Table 3: Pearson correlation matrix among P solubilizing bacteria (PSB), soil physics and soil chemistry in the acidic soil (0-20 cm layer) of oil palm rhizosphere

Characteristics	Sand	Silt	Clay	pH H_2O	N-total	C-organic	CEC	K	Ca	Mg	P_2O_5	Available P bray II	PSB
Sand	1	-0.78	-0.92	0.82	0.53	-0.40	-0.93	-0.68	0.22	0.08	-0.25	-0.76	-0.64
Silt		1.00	0.48	-0.90	-0.46	0.09	0.70	0.82	-0.30	-0.20	0.64	0.87	0.41
Clay			1.00	-0.60	-0.47	0.51	0.87	0.44	-0.13	0.01	-0.05	0.52	0.65
pH H_2O				1.00	0.24	0.03	-0.72	-0.87	0.59	0.51	-0.36	-0.97	-0.28
N-total					1.00	-0.20	-0.38	0.04	-0.30	-0.52	-0.11	-0.05	-0.36
C-organic						1.00	0.65	0.24	0.25	0.34	0.38	0.03	0.94*
CEC							1.00	0.75	-0.28	-0.14	0.41	0.72	0.84*
K								1.00	-0.57	-0.55	0.65	0.95	0.48
Ca									1.00	0.96	0.01	-0.60	0.09
Mg										1.00	0.02	-0.58	0.22
P_2O_5											1.00	0.46	0.85*
Available P bray II												1.00	0.32
PSB													1.00

*Correlation is significant at the 0.05 probability level (2-tailed)

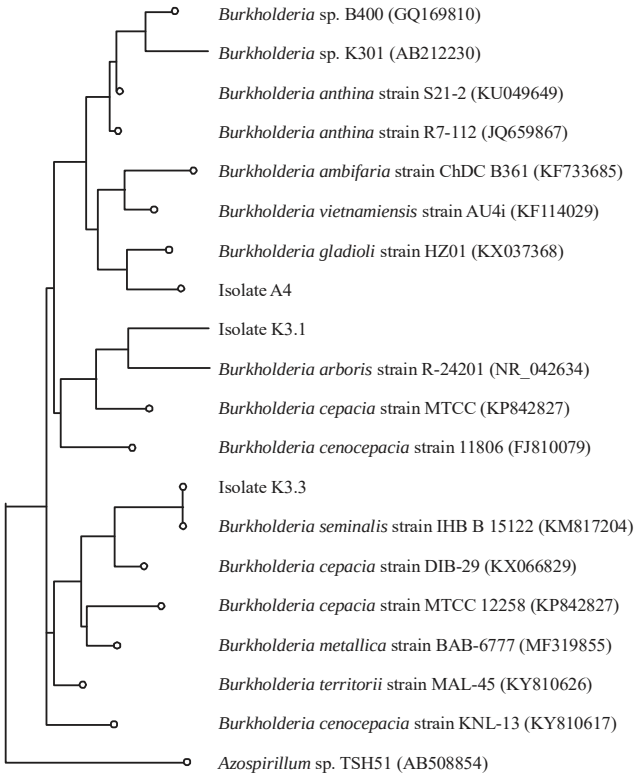

Fig. 2: Phylogenetic tree showing the relationships between the PSB from oil palm rhizosphere in this study and their closest phylogenetic relatives based on 16S rRNA gene sequencing

Molecular identification: Based on the 16S rRNA sequence analysis, all the strains were identified in the genus of *Burkholderia* spp. (Fig. 2). Isolates A.4, K3.1 and K3.3 were identified as *Burkholderia gladioli*, *Burkholderia arboris* and *Burkholderia seminalis*, respectively. Previous reports also described that some *Burkholderia* spp. were efficient as phosphate solubilizers by producing organic acids[28,29]. *Burkholderia* was known as N fixation bacteria and has the antifungal ability[30,31]. Moreover, it was efficient bacteria used as a biological fertilizer on agricultural land[32,33].

CONCLUSION

In the present study, rhizosphere soil samples from oil palm were screened for the isolation of PSB. Based on the sequencing results, isolates K3.1, A4 and K3.3 were closely related to *Burkholderia arboris*, *Burkholderia gladioli and Burkholderia seminalis*, respectively.

SIGNIFICANCE STATEMENT

These results discover the existence of the important PSB living in the rhizosphere of oil palm growing in acid soil. It may help the researchers do the possible studies on utilization of these potential microbes in order to improve soil fertility, particularly for the phosphorus content in acid soil. Thus, a new theory on the existence of PSB in relation to soil-phosphorous source and possibly future application as soil-applied biofertilizer, may be arrived at.

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