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Antagonistic Effect of Two Indigenous Phosphate Solubilizing Bacteria, *Burkholderia contaminans* PSB3 and *Acinetobacter baumannii* PSB11 Isolated from Different Crop Soils

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Phosphorus is the most important key element in the nutrition of plants. Although P is abundant in soils, it is a major limiting factor for plant growth as it is in an unavailable form for roots uptake. Phosphate solubilizing bacteria (PSB) has ability to convert insoluble form of P to an available form. This study was aimed at screening and characterizing phosphate-solubilizing bacteria from manure and different soils and to ascertain a potential benefit to use mixed cultures to improve P solubilization. A total of 12 PSB colonies were isolated on Pikovskaya's agar medium containing tricalcium phosphate. Out of 12 bacterial isolates, 2 isolates showed high phosphate solubilization index (2.17 and 1.83, respectively) were selected for further study. Based on the 16S rRNA gene sequence analysis, PSB3 was closely related to *Burkholderia contaminans* (99%), and PSB11 was closely related to *Acinetobacter baumannii* (99%). The mean P dissolved in liquid cultures of PSB3 and PSB11 in a 14-day incubation were 96.7 and 39.3 mg l^{-1}, respectively. Mixed inoculation of *B*. *contaminans* PSB3 and *A*. *baumannii* PSB11 could not increase the solubilization activity significantly, suggesting there is antagonistic behavior of one isolate towards another. As the interaction of these two isolates may be antagonistic, co-inoculation of these bacteria for P solubilization is not recommended. However, further study is needed to confirm these results.

Key words: agriculture, phosphate-solubilizing bacteria, phosphorus, pure and mixed cultures, soils

Fosfor merupakan elemen kunci terpenting dalam nutrisi tanaman. Meskipun P berlimpah di tanah, P merupakan faktor pembatas utama untuk pertumbuhan tanaman karena P berada dalam bentuk yang tidak tersedia, tidak langsung dapat diserap akar. Bakteri pelarut fosfat (BPF) memiliki kemampuan untuk mengubah bentuk P yang tidak larut menjadi bentuk yang tersedia. Penelitian ini bertujuan untuk menapis dan mengkarakterisasi bakteri pelarut fosfat dari pupuk kandang dan tanah yang berbeda serta memastikan manfaat potensial penggunaan kultur campuran untuk meningkatkan kelarutan P. Sebanyak 12 koloni BPF diisolasi menggunakan medium agar Pikovskaya yang mengandung trikalsium fosfat. Dari 12 isolat bakteri, 2 isolat yang menunjukkan indeks kelarutan fosfat yang tinggi (masing-masing 2,17 dan 1,83) dipilih untuk penelitian lebih lanjut. Berdasarkan analisis urutan gen 16S rRNA, PSB3 berkerabat dekat dengan *Burkholderia contaminans* (99%), dan PSB11 berkerabat dekat dengan *Acinetobacter baumannii* (99%). Rata-rata P terlarut dalam kultur cair PSB3 dan PSB11 setelah inkubasi 14 hari masing-masing adalah 96,7 dan 39,3 mg l^{-1}. Kultur campuran *B. contaminans* PSB3 dan *A. baumannii* PSB11 tidak menunjukkan peningkatan aktivitas pelarutan P secara signifikan. Hal tersebut mengindikasikan perilaku antagonis di antara kedua isolat. Berdasarkan hal tersebut, ko-kultur kedua bakteri untuk pelarutan P tidak dianjurkan. Untuk konfirmasi hasil tersebut diperlukan studi lebih lanjut.

Kata kunci: bakteri pelarut fosfat, fosfor, kultur murni dan campuran, pertanian, tanah

Phosphorous (P) is one of the major essential nutrient for plant growth and development. However, even though soils may contain high number of total P, plant P availability is often reported to be limited, particularly in tropical soils (Collavino *et al*. 2010; Santana *et al*. 2016). In agricultural practices, P fertilizer is applied to add P into the soil in order to fulfil the crops demands for P. However, high P content in soil given by fertilizer cannot be totally available for plant roots due to P in fixed and precipitated forms (Fernández *et al*. 2007). This not only increases production costs, but also leads to environmental pollution (Wang *et al*. 2018).

P uptake by crop can be improved by enhancing P solubility in soil solution and/ or decreasing P fixation in soil. The unavailable P compounds can be made available for the plant by phosphate solubilizing microorganisms (PSM). Among the whole microbial population in soil, bacteria are the predominant microorganisms that solubilize phosphate mineral in nature, as compare to fungi (Sharma *et al*. 2013). Phosphate solubilizing bacteria (PSB) could be referred as the most important bacteria which can

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convert the insoluble P to an accessible form for plant growth (Yousefi *et al*. 2011). Strains from bacterial genera such as *Pseudomonas*, *Mycobacterium*, *Micrococcus*, *Enterobacter*, *Bacillus*, *Erwinia*, *Azotobacter*, *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Acinetobacter*, *Flavobacterium*, *Klebsiella*, and *Micrococcus* have been resported as efficient PSB in the soil (da Costa *et al*. 2015; Paul and Sinha 2017; Pereira and Castro 2014; Sharma *et al*. 2013). PSB can be applied using two approaches, i.e. single culture approach where PSB can be used alone or mixed culture approach, often called co-inoculation, where PSB are used along with other beneficial rhizospshere microorganisms (Khan *et al*. 2007).

PSB could bring about the insoluble forms of the phosphate into soluble forms via various mechanisms. They may decrease the pH of the soil by the producing organic (gluconic acid) and mineral acids (Chen *et al*. 2006), alkaline phosphatases (Rodríguez and Fraga 1999), phytohormones and H^+ protonation (Xiao *et al*. 2017), anion exchange, chelation and siderophores production which promote P solubilization in soil (Sugihara *et al*. 2010). The objectives of this study were to isolate and evaluate the effect of pure and mixed inoculation of two phosphate solubilizing bacterial isolates upon phosphate solubilization efficiency in Pikovskaya's broth medium and to ascertain a potential benefit to use mixed cultures to improve P solubilization.

MATERIALS AND METHODS

Study Area and Soil Collection. The soil samples were collected from the fields of tomato (*Solanum lycopersicum*), Chinese cabbage (*Brassica rapa* var. *pekinensis*), chilli (*Capsicum annum*), leek (*Allium fistulosum*) in Semarang Regency, Indonesia. They were taken from depth of 10 cm using a metal ring (5 cm). Sample was also collected from manure. Three composite samples were taken for each site. A composite sample was created from combining 5 individually collected samples.

Isolation, Purification and Solubilization Index of PSB. One g of each samples were suspended in 10 ml sterile physiological salt solution. Then, 1 ml was plated on Pikovskaya's agar medium (Pikovskaya 1948) containing 5 g l^{-1} tricalcium phosphate (Merck) as the phosphate source by using pour plate method. The composition of Pikovskaya's agar medium was 10 g l^{-1} glucose (Merck), 0.5 g l^{-1} yeast extract (Merck), 0.5 g l^{-1} $(NH_4)_2SO_4$ (Merck), 0.2 g l^{-1} KCl (Merck), 0.1 g l^{-1} $MgSO_4 \cdot 7H_2O$ (Merck), 0.0001 g l^{-1} $MnSO_4 \cdot H_2O$ (Merck), 0.0001 g l^{-1} $FeSO_4 \cdot 7H_2O$ (Merck) and 20 g l^{-1} agar (Merck). The inoculated plates were incubated at 30 C for 48 h in an incubator (Memmert, Germany). After incubation, single colonies from each sample which grew and showing clear zones on plates were picked and restreaked onto fresh Pikovskaya's agar medium using quadrant streak method. This procedure was repeated until pure culture was obtained. The solubilization index (SI) was calculated from the measurements recorded using a transparent ruler after 7 days of growth of pin point inoculation on Pikovskaya's agar medium at 28 C. The solubilization index was calculated as the ratio of halo zone diameter and colony diameter (Liu *et al*. 2015).

Phosphate Solubilization Activity. Based on solubilization index on solid medium, two isolates with the highest index were selected and inoculated into 50 ml preculture Pikovskaya's broth medium and incubated at 30 C for 48 h at 120 rpm on a cooled orbital incubator (Gallenkamp, England). These two isolates were evaluated for P solubilization activity in pure cultures and three mixed cultures (10% v/v) in 100 ml Pikovskaya's broth medium. The ratios of the two isolates were 1:1, 1:2, and 2:1. These were done in triplicate. The suspensions were sampled at day 0, 7, and 14 post-incubation for determination of P concentrations and cell numbers. The P concentration was determined by the vanado-molybdophosphoric yellow color method with a spectrophotometer (UVmini-1240 UV-VIS Spectrophotometers, Shimadzu) at 430 nm. The vanadomolybdate reagent was a solution of 25 g l^{-1} $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (Merck) and 1.25 g l^{-1} NH_4VO_3 (Merck), and 250 ml concentrated HNO_3 (Merck). The density of each isolate was checked by serial dilution by pour plate method for cfu l^{-1}. Bacterial growth was estimated by measuring absorbance at 660nm using a spectrophotometer (UVmini-1240 UV-VIS Spectrophotometers, Shimadzu).

Identification of Phosphate Solubilizing Bacteria Using 16S rRNA Gene. Two PSB isolates were grown in Luria-Bertani (LB, Merck) slant medium at 28 C for 18 h. Genomic DNA from isolates were extracted using Quick-DNA fungal/bacterial miniprep kit (Zymo Research). The 16S rRNA gene with the universal bacteria was amplified using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). All reactions were carried out in reaction mixture was 25.0 µl volumes containing 12.5 µl master mix

(FideliTaq), 1 µl of each primer (10 µM), 1 ml BSA (Biolabs, 10 mg ml^{-1}), 1 µl genomic DNA, and 8.5 µl ddH_2O (MP Biomedicals). The following cycle conditions were used: an initial denaturation at 94 C for 5 min, followed by 35 cycles of 94 C for 30 s, annealing at 54 C for 30 s, and elongating at 72 C for 30 s. The reaction was completed with an extention step at 72 C for 10 min. PCR was done using a thermal cycler (Eppendorf Nexus GSX1). The amplified products were sent to 1st BASE Laboratories for further sequencing using primers 27F and 1492R with an expected amplicon of ~1400 bp. The nucleotide sequences were aligned and compared with those available standard sequences in the GeBank database (https://www.ncbi.nlm.nih.gov/) using BLAST. A phylogenetic tree was constructed using Mega software (Kumar *et al*. 2018) with sequences from closely related strains retrieved from Genebank. The sequences were aligned by ClustalW to reconstruct a phylogenetic tree using the Maximum Composite Likelihood method (Tamura *et al*. 2004) and Neighbor-Joining method (1000 bootstrap replicates). The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers MT622527 and MT622528.

Statistical Analysis. Microsoft Excel 2016 was applied to data analysis. SPSS 22.0 was employed for all statistical analyses and statistical significance was identified when $P \leq 0.05$.

RESULTS

Isolation, Characterization and Screening of PSB Isolates. In this study, there were twelve PSB isolates obtained from soil samples of crop plants and manure (Table 1). The size of P solubilizing halos and colonies of these twelve isolates were different, indicating that bacterial isolates exhibited various P solubilizing activities. The ratio of halo zone to colony zone on Pikovskaya's agar medium containing tricalcium phosphate of isolates ranged from 1.14 to 2.17 (Table 1). The results showed that among the twelve PSB isolates, PSB3 and PSB11 were the most efficient phosphate solubilizing isolates with the solubilization index (SI) values of 2.17 and 1.83 respectively, and therefore selected for further identification and quantification studies. The smallest SI of 1.14 were detected from PSB7 and PSB12 isolates.

Identification of PSB Isolates. Based on 16S rRNA sequences and alignment with database deposited in NCBI GenBank, the isolates were found to belong to *Burkholderia* and *Acinetobacter* species. PSB3 isolate showed homology with partial sequence of *Burkholderia contaminans*, whereas PSB11 isolate was identified as *Acinetobacter baumannii* (Table 2). Two PSB3 and PSB11 sequences have higher than 98% identity with the queried sequence. Fig 1 also showed the relationship of the isolates with their closest relatives from NCBI GenBank.

Phosphate Solubilizing Capability of PSB in Pure and Mixed Cultures. The calcium phosphate solubilizing activities of PSB3 and PSB11 in pure and mixed cultures during the 7 and 14 days cultivation were shown in Table 3. The amount of phosphates solubilized by PSB3 isolate was showed to be significantly (P<0.05) higher than PSB11 and positive controls (*Pseudomonas putida* and *P. aeruginosa*) during the 7 and 14 days of incubation time. PSB3 isolate solubilized 82.9 and 96.7 mg l^{-1} of P, while PSB11 solubilized 10.8 and 39.3 mg l^{-1} of P after 7 and 14 days of incubation, respectively.

Table 3 also showed the cell density of pure and mixed cultures in Pikovskaya's broth medium during the 14 days cultivation. All pure and mixed cultures grew well, but there were not significantly different between treatments for colony number (P>0.05). Significant differences were observed in the P solubilization of pure and mixed cultures as shown in Table 3. The three different combinations of mixed cultures of *B. contaminans* PSB3 and *A. baumannii* PSB11 isolates showed significantly (P<0.05) lower phosphate solubilization when compared with pure *B. contaminans* PSB3 inoculation after 7 and 14 days of incubation (Table 3).

DISCUSSION

Phosphorus in soil is important for plant development, and the lack of P limits plant growth. The selection of highly efficient PSB will practically increase phosphorus in plant rhizosphere. Various PSB have been isolated from different soil and rhizosphere (Yu *et al*. 2011; Majeed *et al*. 2015). Therefore, PSB can be regarded as plant growth-promoting rhizobacteria, which are widely considered as alternatives to common biofertilizers (Pathak *et al*. 2019).

In the present study, all these 12 isolates showed phosphate solubilization activity as indicated by halo zone formation on Pikovskaya's agar medium containing tricalcium phosphate. The halo zone formation around the bacterial colonies could be due to

Table 1 Solubilization index of PSB isolates from crop soils and manure on Pikovskaya's agar medium

Sample	Isolate code	Halo zone diameter (mm)	Colony diameter (mm)	Solubilization index
Chinese cabbage	PSB1	5	4	1.25
Chinese cabbage	PSB2	6	4	1.50
Chinese cabbage	PSB3	13	6	2.17
Chilli	PSB4	5	4	1.25
Leek	PSB5	6	4.5	1.33
Leek	PSB6	5	4	1.25
Manure	PSB7	4	3.5	1.14
Tomato	PSB8	6	5	1.20
Tomato	PSB9	7	5	1.40
Tomato	PSB10	6	4.5	1.33
Tomato	PSB11	11	6	1.83
Tomato	PSB12	4	3.5	1.14

Table 2 Molecular characterization of PSB3 and PSB11 isolates by 16S rRNA sequences

Isolate (GenBank Accesion no.)	Most closely related organism (16S rRNA Accesion no.)			Sequence query coverage (%)
	Species	Strain	% similarity	
PSB3 (MT622527)	*Burkholderia contaminans* (NR104978)	J2956	99.3	98
PSB11(MT622528)	*Acinetobacter baumannii* (NR117620)	ATCC19606	99.8	98

Table 3 The P solubilization (mg l^{-1}) of pure and mixed culture of PSB (n = 3).

Isolate	Incubation period (days)		Cell number after 14 days (CFU $\times 10^{11}$)
	7	14	
PSB3	82.9 ± 13.46 b	96.7 ± 14.90 c	1.23 ± 0.07 b
PSB11	10.8 ± 1.77 a	39.3 ± 0.40 b	0.96 ± 0.18 ab
Mixed culture 1	15.1 ± 0.97 a	28.7 ± 2.57 ab	0.62 ± 0.06 ab
Mixed culture 2	20.3 ± 0.57 a	22.4 ± 1.39 ab	0.82 ± 0.34 ab
Mixed culture 3	18.7 ± 1.19 a	40.1 ± 7.14 b	1.00 ± 0.03 ab
P. poetida	9.6 ± 2.19 a	13.2 ± 1.83 a	0.79 ± 0.04 a
P. aeruginosa	23.6 ± 1.27 a	27.5 ± 1.14 ab	0.95 ± 0.30 ab

Means followed by a common letter are not significantly different between treatment by the Tukey tests at the 0.05 level of significance. Mixed culture 1, 2, 3 are PSB3 and PSB11 at an inoculum ratio of 1:1, 1:2, and 2:1, respectively.

the solubilizing of tricalcium phosphate by organic acids, polysaccharides, or phosphatases secreted from PSB isolates (Alori *et al*. 2017; Illmer and Schinner 1995; Pande *et al*. 2017; Paul and Sinha 2017).

In this study, there was no significant difference between pure and mixed cultures in bacterial cell number. Therefore, P solubilization activity was not associated with bacterial cell number. The main mechanism of phosphate solubilization is the production of some organic acids and production of these organic acids results in the lowering of pH of the medium (Chen et al. 2006; Chen et al. 2016; Li et al. 2016; Song et al. 2008). The lowering in pH of the medium suggests the release of organic acids by the P-solubilizing microorganisms via the direct oxidation pathway that occurs on the outer face of the cytoplasmic membrane (Maliha et al. 2004; Babu-Khan et al. 1995). Nevertheless, organic acids production and identification as well as pH of the culture medium during the 14 days cultivation were not determined in this study.

From 12 isolates, two of PSB (PSB3 and PSB11) were selected for further sudies; they showed a greater solubilizing activity than that of the control

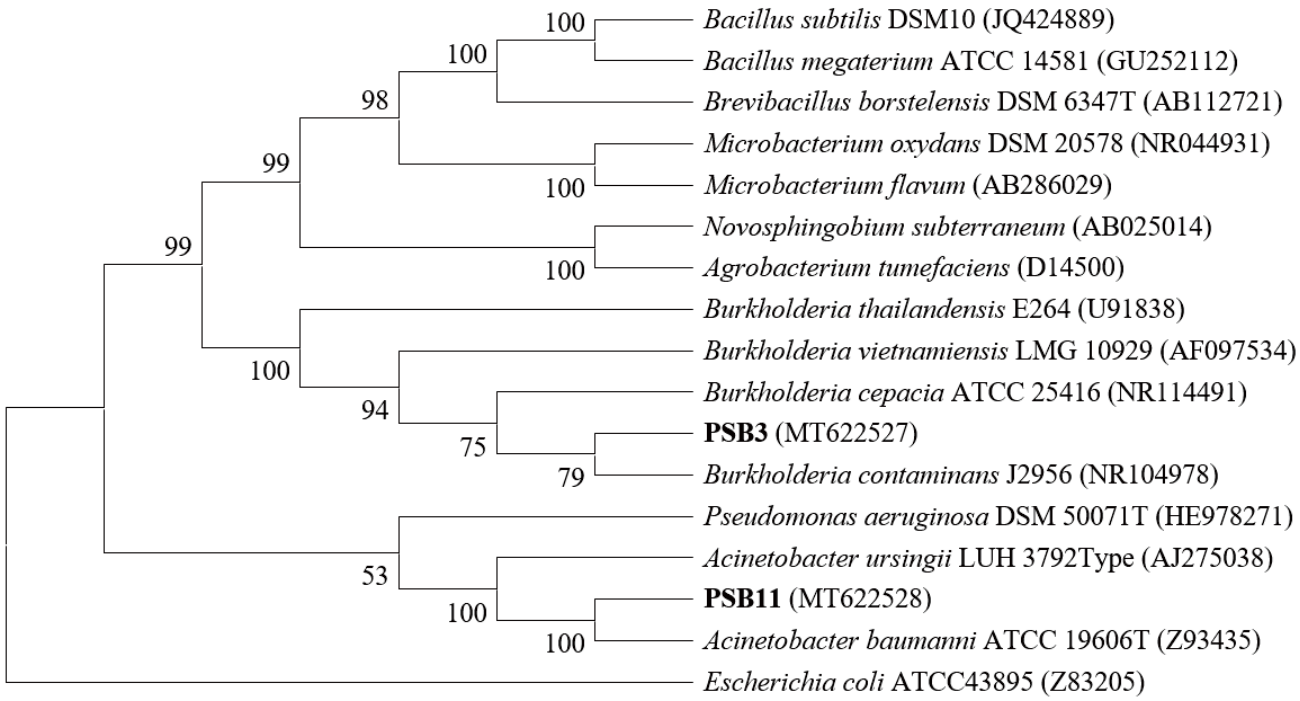

Fig 1 Phylogenetic tree showing the relationships between the phosphate-solubilizing bacteria (PSB) isolates in this study and their closest phylogenetic relatives based on 16S rRNA gene sequences (accession numbers are given in parentheses). Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points. The tree was rooted using *Escherichia coli* ATCC43895 (Z83205) as the outgroup.

Pseudomonas putida and *P. aeruginosa* under in vitro conditions. These isolates were identified as *B. contaminans* (PSB3) and *A. baumanii* (PSB11) by PCR amplification and partial nucleotide sequencing of the 16S rRNA. The strain PSB3 is part of the so-called *B. cepacia* complex (Vandamme and Dawyndt 2011), which include at least 20 species. *Burkholderia cepacia* complex known as part of the soil microbial community (Baldwin *et al*. 2007; Rojas-Rojas *et al*. 2018). Previous reports also indicate that *B. contaminans* and *A. baumanii* possess the ability of solubilizing phosphate (Ghosh and Mandal 2020; Pande *et al*. 2019; You *et al*. 2020).

Compared with single inoculation of *B. contaminans*, co-inoculation of *B. contaminans* PSB3 and *A. baumanii* PSB11 showed lower P solubilization activity. These results contrasted with the previous reports. For example, Park *et al*. (2016) found a synergistic effect of co-inoculation of *B. anthina* PSB-15 and *Enterobacter aerogenes* PSB-16 in liquid medium. Teymouri *et al*. (2016) showed a positive effect on total P solubilized by mixed cultures of PSB (*Bacillus*, *Streptomyces*, and *Pseudomonas*) as solubilizing efficiency increased by 373 mg l^{-1}. Braz and Nahas (2012) also showed a synergistic action of the fungus *Aspergilus niger* and the bacterium *B. cepacea* in co-culture. Our results suggest that PSB capable of antagonizing the activity of other PSB as found by other researchers (Lazzarini *et al*. 2000; Paul and Sinha 2017; Trivedi *et al*. 2008; Zhao *et al*. 2014; Rojas-Rojas *et al*. 2018; Rojas-Rojas *et al*. 2019; Vial *et al*. 2007). Phosphate solubilizing *Actinobacteria* able to produce a large number of secondary metabolites, many of which possess antibacterial activity (Lazzarini *et al*. 2000). *Burkholderia contaminans* is a strain from the *B. cepacia* complex (Vandamme and Dawyndt 2011), which members of *B. cepacia* complex are known for producing a wide range of antimicrobial compounds that inhibit bacteria, yeast and fungi including other *B. cepacia* complex strains (Rojas-Rojas *et al*. 2018; Rojas-Rojas *et al*. 2019; Vial *et al*. 2007) as well as by lowering pH (Zhao *et al*. 2014).

Phosphate-solubilizing antagonistic bacteria can be useful for enhancing plant P contents as well as supression of disease infection. In previous study, antagonistic rhizobacteria having potential for *in vitro* phosphate solubilization were reported for disease supression of bacterial leaf blight and growth promotion of rice (Rasul et al. 2019; Yasmin et al. 2016). The findings of the current study highlighted that PSB from soil could be easily isolated and may be exploited as biofertilizer and biocontrol agent to improve the crop

productivity. However, evaluation the potential of antagonistic *B. contaminans* and *A. baumanii* for disease supression and growth promotion need to be study further.

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