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

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 reported 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 rhizosphere 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 (Rodriguez 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 (NH₄)₂SO₄ (Merck), 0.2 g l⁻¹ KCl (Merck), 0.1 g l⁻¹ MgSO₄·7H₂O (Merck), 0.0001 g l⁻¹ MnSO₄·H₂O (Merck), 0.0001 g l⁻¹ FeSO₄·7H₂O (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 (NH₄)₆MoO₄·4H₂O (Merck) and 1.25 g l⁻¹ NH₄VO₃ (Merck), and 250 ml concentrated HNO₃ (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
Identification 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).

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

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

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

<table>
<thead>
<tr>
<th>Isolate (GenBank Accession no.)</th>
<th>Most closely related organism (16S rRNA Accession no.)</th>
<th>Sequence query coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSB3 (MT622527)</td>
<td><em>Burkholderia contaminans</em> (NR104978) J2956</td>
<td>98</td>
</tr>
<tr>
<td>PSB11 (MT622528)</td>
<td><em>Acinetobacter baumannii</em> (NR117620) ATCC19606</td>
<td>98</td>
</tr>
</tbody>
</table>

Table 3 The P solubilization (mg 1⁻¹) of pure and mixed culture of PSB (n = 3).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Incubation period (days)</th>
<th>Cell number after 14 days (CFU ×10¹⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>PSB3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSB11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed culture 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed culture 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed culture 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. poetida</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 studies; they showed a greater solubilizing activity than that of the control...
**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 *Aspergillus 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 suppression of disease infection. In previous study, antagonistic rhizobacteria having potential for *in vitro* phosphate solubilization were reported for disease suppression 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 \textit{B. contaminans} and \textit{A. baumanii} for disease suppression and growth promotion need to be study further.

REFERENCES


