

Research Article

Characterization of phosphate solubilizing bacteria isolated from Pb contaminated soils and their potential for dissolving tricalcium phosphate

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Abstract: Phosphorus solubilizing bacteria (PSB) enhances P availability in soils through dissolving inorganic P pools. The characteristics of PSB and the potential of three Pb tolerant phosphate solubilizing rhizobacteria, i.e. *Pseudomonas* sp., *Bacillus* sp., and *Actinomyces* sp. were evaluated. PSB were isolated from soil samples contaminated with 300 and 500 mg Pb/kg after incubation for 30 days. Phosphate solubilizing bacteria were screened on for phosphate solubilisation ability in Pikovskaya agar medium (PA). In addition, two of the three identified PSB strains (*Pseudomonas* sp. and *Bacillus* sp.) were characterized for their ability to solubilize tricalcium phosphate in Pikovskaya broth (PB) and also were examined their growth during culture medium incubation. The isolates exhibited different phosphate solubilization index, ranging from 1.87 to 2.98. *Pseudomonas* sp. had the highest ability to solubilize tricalcium phosphate: 9.82 mg P/ L and 12.23 mg P/ L in Pikovskaya broth following the addition of 4 mg Pb/L and 2 mg /Pb L, respectively.

Keywords: P solubilization, Pb contaminated soils, phosphate solubilizing bacteria, Pikovskaya medium

Introduction

Excessive use of fertilizers and pesticides, industrial activity, and mining can result in heavy metal contamination of soils (Bolan et al., 2003). Lead (Pb) is a major contaminant which results in severe environmental and human health hazards (Hoaet al., 2007). Subowo et al. (2005) reported that the Pb content of rice field soils around the industrial area in west Jakarta ranged from 206 to 449 mg/ kg, while the Pb content in the rice field soils was 100 mg/kg¹ (Dawes, 1990). Elevated Pb levels in the soil may inhibit soil microbial activity. Saraswati (2004) reported decreased soil respiration and microbial carbon as well as alteration of beneficial microbial communities in Pb contaminated soils. Among the beneficial soil microbia, phosphate solubilizing bacteria (PSB), which play an important role in enhancing phosphorous availability, is most sensitive to Pb. PSB groups include *Pseudomonas*, *Micrococcus*, *Bacillus*, *Flavobacterium*, *Rhizobium*, *Enterobacter*, *Azospirillum* and *Erwinia* (Rodriguez and Fraga, 1999) and their response to

contamination may be variable. The objectives of this research were to investigate characteristics of phosphate solubilizing bacteria (PSB) isolated from soils contaminated with Pb and assess their potential for dissolving tricalcium phosphate in pikovskaya broth.

Materials and Methods

Fifteen kilograms of composite soil samples of Udipsamment (0-20 cm) were randomly collected from three rice fields (each of which is 0.5 ha) at Dasan Geres village, West Lombok regency. The samples were air-dried and ground to pass through a 2 mm hole size sieve for laboratory analysis. The soil characteristics were as follows: soil pH: 6.3, organic C: 1.01%, CEC:13.87 cmol_c/kg Bray I P₂O₅:19.2 mg/kg, total Pb: 12.38 mg/kg, and loamy sand texture. Treatments tested in this experiment were application of 300 mg Pb/kg and 500 mg Pb/kg supplied as lead nitrate. Each treatment used five kilograms or the air-dried soil and put into a plastic pot (25 x 40 cm) to which 100 kg/ha SP-36 fertilizer and 5 t/ha of

organic manure were added and mixed thoroughly. Each treatment was replicated thrice and incubated for 30 days.

PSB from the soil contaminated with Pb were isolated by serial dilutions (10^{-1} up to 10^{-5}) and spread plate method using pikovskaya agar medium (10 g glucose; 5 g Ca_3PO_4 ; 0.5 g $(\text{NH}_4)_2\text{SO}_4$; 0.2 g KCl; 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5 g yeast extract, 20 g agar, 1000 mL distilled water, and supplemented with few?? of MnSO_4 and FeSO_4 ; pH 7). To prevent the growth of soil fungi, 10 mg/L fungicidin were added after sterilizing. PSBs were isolated according to the method described by Niswati et al. (2007).

Ten gram soil sample contaminated with either 300 mg Pb/kg or 500 mg Pb/kg⁻¹ was put into 90 ml of sterile saline solution (8.5 mg NaCl /L distilled water) to give 10^{-1} dilution and was thoroughly shaken. One ml of the suspension was transferred to 9 ml of the saline solution to form 10^{-2} . Similarly 10^{-3} , 10^{-4} and 10^{-5} serials were made for each soil sample. Aliquots of 0.1 ml of each dilution were spread on Pikovskaya agar medium containing calcium phosphate and incubated at room temperature (28-30°C) for 5 days. Colonies indicating halo zones with a large relative diameter were picked and purified by three times subculture method on Pikovskaya agar medium for studying their characteristics. The morphology, gram characteristics, and pigment production of the selected PSBs were examined. In particular, assimilation of carbon (glucose, lactose, sucrose, manitol and fructose), gelatine hydrolysis and starch hydrolysis were evaluated. Production of catalase, urease, indole acetic acid, and H_2S , as wells as nitrate reduction were tested. Furthermore, type strains of the PSB based on a specific medium for each strain were also determined.

PSB's ability in dissolving $\text{Ca}_3(\text{PO}_4)_2$ as the single P source in the 2 mg Pb/L spiked Pikovskaya agar medium (PA) was determined according to the method described by Premono et al. (1996). Their capabilities were indicated with P solubilization index (halo zone + colony diameter/colony diameter) after incubation for 3 x 24 hours. P solubilization by PSB was quantified using tricalcium phosphate [$\text{Ca}_3(\text{PO}_4)_2$] (5 mg/L) on pikovskaya broth (PB) containing of various Pb concentration (2 and 4 mg/L), supplied as lead nitrate. Evaluation of P solubilization ability of each strain of PSB was conducted as follows: Pb was added to a 250 ml erlenmeyer filled with 100 mL of pikovskaya broth inoculated with the PSB strain (1 mL/L bacterial suspension (ca. 1×10^9 /cfum) and incubated at room temperature (28-30°C) for 7 days on a shaker at 125 rpm. The

solubilized P on each PB was measured after 7 days. Phosphorous content was measured using a spectrophotometer by the phosphomolybdate method (Rodrigues and Fraga, 1999). pH and population growth of PSB were measured every two days (e.g., 1, 3, 5 and 7 days after incubation). The growth pattern of PSB was predicated by measuring the optical density (OD) with a 600 nm wave length spectrometer

Results and Discussion

There were many bacteria forming halo zones found on soil contaminated with 300 or 500 mg Pb/kg. However, there were only three PSB strains which showed high relative P solubilization index in PA, implying that there were not many genus of Pb tolerant PSB (Table 1). The strains were putatively identified as *Pseudomonas* sp., *Bacillus* sp., and *Actinomyces* sp. (Fig. 1), Raone (1999) also isolated Pb resistant bacteria (*Pseudomonas marginalis* and *Bacillus megaterium*) from heavy metal contaminated soils. Likewise, Park et al. (2010) isolated PSB from Pb-contaminated and P-amended soils, which were identified as *Enterobacteriasp* and *Pantoeasp*. Most of resistant bacteria on heavy metal pollutants are gram negative (Geesey and Jang, 1990), produced extracellular polymers (polysaccharides and proteins) to decrease Pb mobility through formation of Pb-organic complex (Park et al., 2011) and produced catalase (Joseph et al., 2007), which is consistent with the present results (Table 1).

Phosphat Solubilization

Characterization of PSB with respect to P solubilization index, solubilized P and medium pH change are presented in Table 2. The PSB ability in dissolving inorganic P (tricalcium phosphate as a single P source) in PA was qualitatively estimated with the P solubilization index (total halo zone + colony diameter/ colony diameter) (Premono et al., 1996). In the present study, the index created by the three identified PSB strains were different, implying differential ability for P solubilization. The largest index was noted for *Pseudomonas* sp., followed by *Bacillus* sp. and *Actinomyces* sp. Furthermore, *Pseudomonas* sp. and *Bacillus* sp. were selected to be tested their ability for dissolving inorganic P in the PB containing of various Pb concentrations (2 and 4 mg/L of Pb).

Based on solubilized P in PB, it can be deduced that the ability of *Bacillus* sp. in

solubilizing inorganic P was lower than that of *Pseudomonas* sp. *Bacillus* sp. was only capable of solubilizing 9.72 and 5.65 of P from tricalcium phosphate in the Pb following the addition of 2 and 4 mg Pb L⁻¹ respectively. *Pseudomonas* sp. solubilized 12.23 and 9.82 of P in the PB

following the addition of 2 and 4 mg Pb L⁻¹ respectively. *Pseudomonas* sp. has the highest potential for dissolving P in soils contaminated with Pb. The high potential of the *Pseudomonas* sp. can be attributed to greater production of extracellular polymers and organic acids.

Table 1. Morphological and biochemical test of *Bacillus* sp, *Pseudomonas* sp, and *Actinomyces* sp.

Characteristics	<i>Bacillus</i> sp.	<i>Pseudomonas</i> sp.	<i>Actinomyces</i> sp
Gram reaction	G-	G-	G+
Morphology of colony	rod	rod	Spiral
Pigment production	-	+	+
Carbon source:			
Glucose	+	+	+
• Lactose	+	-	-
• Sucrose	+	-	-
• Manitol	+	-	+
• Fructose	+	-	+
Biochemical test:			
• Catalase activity	+	+	+
• Oxidase	-	-	-
• Urease	+	-	-
• NO ₃ reduction	+	-	+
• H ₂ S production	-	+	+
• Indole production	-	-	+
• Use of citrate	+	+	+
• Starch hydrolysis	+	+	+
• Gelatin hydrolysis	+	-	-

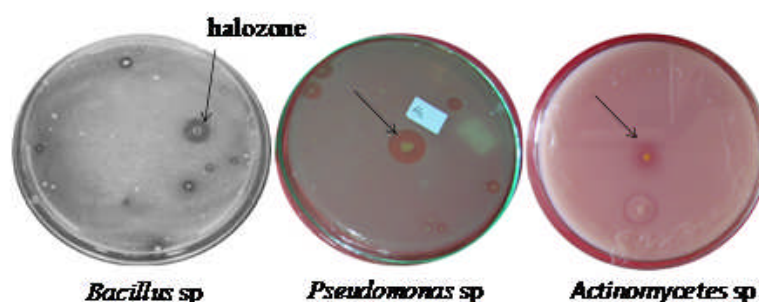


Figure 1. Halozone of P solubilization *Bacillus* sp, *Pseudomonas* sp, and *Actinomyces* sp from soil sample contaminated with Pb metal in PA medium

Park et al. (2011) explained that several strain of soil bacteria produce extracellular polymers to reduce metal toxicity with extracellular sequestration of Pb. Although the P solubilization mechanisms by organic acids are not well understood (Cao et al. 2008), the chelation-mediated mechanisms and the pH decrease are the main process in P solubilization from inorganic P (Sharma et al., 2011).

Furthermore, increasing Pb concentration in the culture medium decreased P solubilization

(Table 2). This can be explained based on the amount of P solubilized by PSB were very likely to be used to immobilize Pb in the culture medium. Cao et al. (2008) reported phosphorus is effective in immobilizing lead (Pb) in contaminated soils through formation of pyromorphite (Pb₅(PO₄)₃). Secondly, Pb toxin effect resulted in the obstruction of bacterial activity in producing organic acids, so that the lower solubilized P were able to occur. Chen et al. (2006) reported the solubilized P in the culture

medium can be affected by a lot of factors covering the composition of the bacterial medium, the pH change of the culture medium, the presence of PSB strain

pH of the medium for both the genus tended to decrease (Figure 2). Furthermore, in the *Pseudomonas* medium either spiked with 2 mg Pb/L or 4 mg Pb/L, the pH continued to decrease from day 1, 3, 5 to day 7. The change magnitude of culture medium pH was attributed to the concentration rate and kind of organic acids released by the PSB genus (Figure 3).

The highest growth of the *Bacillus* sp. in the 2 mg Pb/L spiked PB was obtained at day 1, then

the growth decreased sharply at day 3 and then occurred stagnant to day 7. The *Bacillus* growth in the 4 mg Pb/L spiked medium decline slightly from day 3 to 5 and the decrease drastically at day 7. Furthermore, our result shown that the growth pattern of *Pseudomonas* sp in both of the media was the same as each other, in which their growth tend to gradually decrease from day 1, 3, 5 to 7. The difference of the growth pattern from both of the genus showed the different adaptation capability on Pb contaminated environment. Based on this result, the *Pseudomonas* sp was selected as the more tolerant strain on Pb metal.

Table 2. Halo zone and colony diameter, P solubilization index in Pikovskaya agar (PA) and solubilized P, medium pH in Pikovskaya broth (PB)

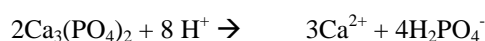
Pikovskaya Medium	PSB strain	Halo zone + colony diameter (mm)	Colony diameter (mm)	P solubilization index (*)	Solubilized P (mg/L)**)	Medium pH **)
PA+2 mg Pb/L	<i>Bacillus</i> sp.	9,72	4	2.43		
	<i>Pseudomonas</i> sp	14,35	5	2.87		
	<i>Actinomycetes</i> sp	5,94	3	1.98		
PB+2 mg Pb/L	<i>Bacillus</i> sp.				9.72	5.10
	<i>Pseudomonas</i> sp				12.23	3.78
PB+4 mg Pb/L	<i>Bacillus</i> sp.				5.65	5.28
	<i>Pseudomonas</i> sp				9.82	4.34

*) after incubation for 3 x24 hours; **) after incubation for 7 days

Based on the solubilized P in PB, it was able to be shown that the ability of *Bacillus* sp. in solubilizing inorganic P was lower than that of *Pseudomonas* sp. *Bacillus* sp. was only capable of solubilizing 9.72 and 5.65 of P from tricalcium phosphat in the Pb following the addition of 2 mg Pb/L and in the addition of 4 mg Pb/L respectively. Meanwhile *Pseudomonas* sp. was able to solubilize 12.23 and 9.82 of P in the PB following the addition of 2 mg Pb/L and the addition of 4 mg Pb/L respectively. This result showed that the *Pseudomonas* sp. has the high potential in dissolving P in soils contaminated with Pb metal. The high potential of the *Pseudomonas* sp. is very likely be attributed to greater production of extracellular polymers and organic acids. Park et al. (2011) explained that several strain of soil bacteria produce extracellular polymers to reduce metal toxicity with extracellular sequestration of Pb.

In addition, it is well known that organic acids play a important role in dissolving inorganic P pools in soils (Park et al., 2010). Although the P solubilization mechanisms by organic acids are not well understood, the chelation-mediated mechanisms and the pH decrease are the main process in P solubilization from inorganic P

(Sharma et al, 2011) . The medium pH decrease resulted from the H dissociation from organic acids. Hydroxyl and carboxyl groups of organic acids chelate cations (Al, Fe and Ca). Equation of P solubilization from a tricalcium phosphate resulted from the pH decrease is given as follow (Bolan et al., 2003).



Furthermore, increasing the Pb concentration in the culture medium resulted in decreasing the solubilized P (Table 2). For these reasons : firstly, a amount of solubilized P by the PSB were very likely to be used to immobilize Pb in the culture medium. Cao et al. (2008) reported phosphorus is effective in immobilizing lead (Pb) in contaminated soils through formation of pyromorphite (Pb₅(PO₄)₃). Secondly, Pb toxin effect resulted in the obstruction of bacterial activity in producing organic acids, so that the lower solubilized P were able to occure. Chen et al. (2006) reported the solubilized P in the culture medium can be affected by a lot of factors covering the composition of the bacterial medium, the pH change of the culture medium, the presence of PSB strain

In relation on the pH change in the PB containing of various concentrations of Pb, the medium pH for both the genus tend to decrease (Figure 2). In the 2 mg Pb/L spiked culture medium in the presence of *Bacillus* sp., the medium pH continued to decrease from day 1 to 7, while the medium pH spiked 4 mg Pb/L decreased from day 1 to 3 and then slightly decreased up to day 7. Furthermore, in the *Pseudomonas* medium either spiked with 2 mg Pb/L or 4 mg Pb/L, the pH continued to decrease from day 1, 3, 5 to day 7. The change magnitude of culture medium pH was able to be attributed to the concentration rate and kind of organic acids released by the PSB genus (Figure 3).

Pattern of the growth of both the genus in the culture medium was as given in Figures 4 and 5,

respectively. The highest growth of the *Bacillus* sp. in the 2 mg Pb/L spiked PB was obtained at day 1, then the growth decreased sharply at day 3 and then occurred stagnant to day 7. The *Bacillus* growth in the 4 mg Pb/L spiked medium decline slightly from day 3 to 5 and the decrease drastically at day 7. Furthermore, our result shown that the growth pattern of *Pseudomonas* sp in both of the media was the same as each other, in which their growth tend to gradually decrease from day 1, 3, 5 to 7. The difference of the growth pattern from both of the genus showed the different adaptation capability on Pb contaminated environment. Based on this result, the *Pseudomonas* sp was selected as the more tolerant strain on Pb metal.

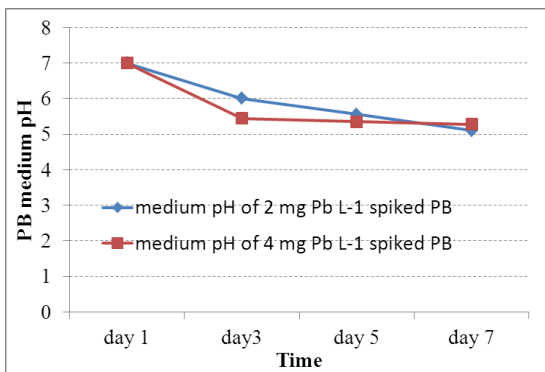


Figure 2. Change of PB medium pH of the *Bacillus* sp. presence

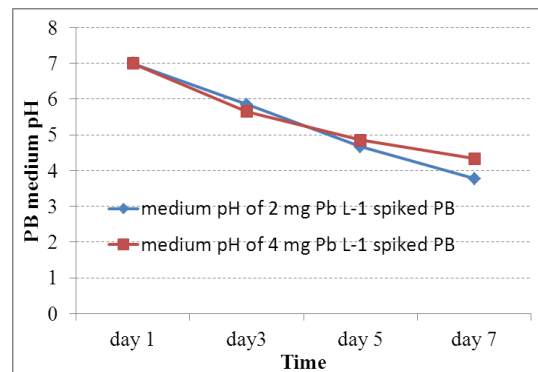


Figure 3. Change of PB medium pH of the *Pseudomonas* sp. presence

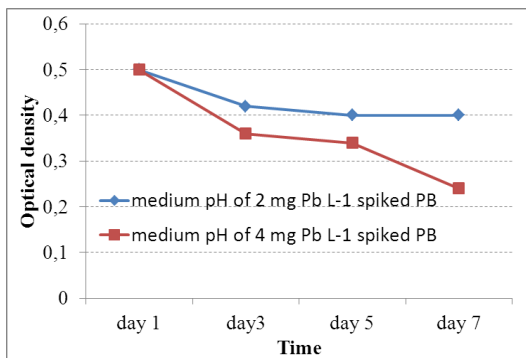


Figure 4. Growth pattern of the *Bacillus* sp. in PB medium

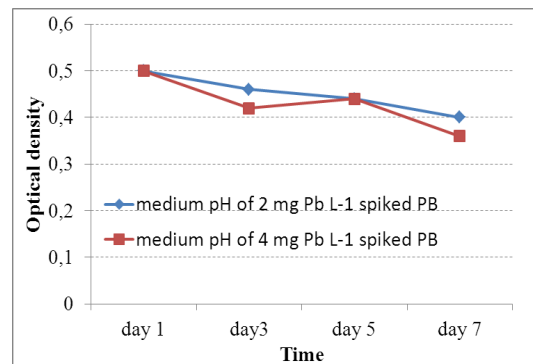


Figure 5. Growth pattern of the *Pseudomonas* sp. in PB medium

Conclusion

Three PSB genus, *Bacillus* sp, *Pseudomonas* sp and *Actinomyces* sp, were found in soils contaminated with Pb metal. In the PA, the largest P solubilization index was created by *Pseudomonas* sp

as following *Bacillus* sp and *Actinomyces* sp. in the range of 2.98, 2.43 and 1.87 respectively. The highest P solubilization was resulted by *Pseudomonas* sp. about 12.23 mg P/L and 9.82 mg P/L in PB following the addition of 2 mg Pb/L or 4 mg Pb/L, respectively. In relation to the

medium pH change, the medium pH of *Pseudomonas* was lower than that of *Bacillus* sp after PB incubation for 7 dys in both the PB. Increasing the Pb concentration in the PB resulted in sharply decreasing the growth of *Bacillus* sp, but slightly in that of *Pseudomonas* sp. Based on the results of this study demonstrated that *Pseudomonas* sp. was able to be selected as the more tolerant PSB strain on Pb metal. Further study is needed to know its potential use as a P-biofertilizer on various crops cultivated in Pb contaminated rice field soils.

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