

Research Article

Isolation of indigenous phosphate solubilizing bacteria from green bean rhizospheres

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Abstract : The use of phosphate-solubilizing bacteria (PSB) as a biological fertilizer of Agricultural land is one solution to overcome problem of phosphate availability for plants. However, often application of a biological fertilizer is ineffective for certain places. The purpose of this study was to obtain *indigenous* phosphate solubilizing bacterial isolates that can be effective in the area of Malang. Samples were collected from *rhizosphere* of green bean plants at three locations in Malang, East Java. The study was conducted to determine the total bacterial population of soil samples, to select the best three bacterial isolates in phosphate solubilizing ability, which is not antagonistic and nonpathogenic for plants, along with observing its potential as a bacterial consortium. The highest total population was found in FHR samples of 1.5×10^{11} CFU / mL. We have selected three bacterial isolates namely SPP1, SPP2 and SPP3. They were not antagonistic to each other and nonpathogenic on mungbean sprouts. They had possibility of producing growth hormone which characterized by an increasing in length of plant and total root length, be compared to controls. Strain SPP2 has shown the highest activity of phosphate solubilization then was selected for 16S rRNA identification. Similarity test of genome sequence of strain SPP2 had 99% similarity with *Pseudomonas plecoglossicida* strain PR19.

Keywords: consortium, indigenous bacteria, Malang, phosphate solubilizing

Introduction

Phosphate (P) is a macro nutrients essential for plant related to essential functions of P as a constituent of Adenosine Tri-Phosphate (ATP) in the plant metabolism. Efforts for increasing the availability of P for plants with fertilization had been made. However, in fact that a number of P fertilizer applied, phosphate availability to plants, especially in acidic mineral soils, is low (Buckman and Brady, 1982). Actually, the total amount of P in the soil is quite high, but the amount of available P to plant is low, only 0.01 to 0.2 mg/ kg soil (Handayanto and Hairiah, 2007). Factors affecting the availability of P include soil pH, the presence of available iron, manganese, calcium, and aluminum as well as the rate of decomposition of organic matter and microorganism activity (Buckman and Brady, 1982). The activities of these microorganisms can be used to overcome the low soil available P using

free soil microbes. They have ability to dissolve P contained in the fertilizer and soil, as well as to help the roots to reach P to be absorbed (Hasanuddin and Bambang, 2004). Some microbes have been known to play important roles in dissolving phosphate are bacteria, fungi and actinomycetes. In the group of bacteria, among others: *Bacillus firmus*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus polymixa*, *Bacillus megatherium*, *Micrococcus*, *Pseudomonas*, *Arthrobacter*, *Achromobacter*, *Flavobacterium*, and *Mycobacterium*. Phosphate solubilizing bacteria (PSB) is a group of bacteria that can dissolve P adsorbed on the surface of iron oxides (Fe-P) and aluminum (Al-P). The bacteria also play a role in the transfer of energy, protein preparation, coenzyme, nucleic acid and other metabolic compounds that can increase the activity of P uptake in plants that lack of phosphate (Subba-Rao, 1995). Those bacteria are some of a group of PSB having high capability as

biofertilizer by dissolving phosphate bound by other elements (Fe, Al, Ca, and Mg), so the phosphate becomes available to plants. Understanding of the rhizosphere nice and microbial community interactions will assist development of inoculants across farming systems with potentially greater consistency in performance and survival, especially the use of indigenous mikroorganisms (Hilda and Fraga, 1999). Nuraini et al. (2015) and Arfarita et al. (2016a) reported that indigenous microrganisms are more sustainable than introduction of microorganisms when applied as a biological fertilizer or for bioremediation. The current study was to isolate indigenous PSB in rizhosphere of green beans (*Vigna radiata* L.) in the area of Malang and to select three bacterial strains with the best ability in solubilizing phosphate.

Materials and Methods

The initial stage was to process a screening and isolation of indigenous phosphate solubilizing bacteria from rhizosphere of green bean plants in the three regions. Three bacteria were selected and then observed their potential as a bacterial consortium that will be used as biofertilizer agents. Its potential was also supported by antagonism and pathogenicity test on green beans sprouts. Identification using 16S rRNA was also conducted on bacteria having the highest phosphate solubilizing activity.

Soil samples

Soil samples were collected in April 2016 from rhizosphere of green bean plants (*Vigna radiata* L.) in the areas of Jambegede (SJG) and Kendalpayak (SKP) and as well as natural forest area in Junrejo (SHR) Malang, East Java. Soil samples were collected by pulling out the plant along with its roots carefully. Plant shoot was cut and then the roots along with the soil were put into plastic bags. They were kept in a cooling box and then immediately taken to the laboratory or stored at 4-8°C to isolate the bacteria.

Estimation of soil bacteria population

One hundred grams of soil samples were suspended in 900 ml physiological saline solution (0.85%) and then arranged serial dilution to 10^{-8} . Estimation of total population of soil bacteria was made by the Standard Plate Count (SPC) method. Based on a preliminary study, a certain dilution series of soil samples were made by taking 50 μ L suspension and spreading to NA (Nutrient Agar) medium on a petridish. Petridishes were then incubated at 37°C over night.

Screening and isolation of phosphate solubilizing bacteria

The initial selection of phosphate solubilizing bacteria was performed by a screening process. Soil samples were processed using spread plate method in a solid Psicovkaya medium, after diluted at specific dilutions of suspensions. Psicovkaya medium of 1000 ml was prepared as follows: Glucose 10 g, NaCl 5 g, G_3 (PO_4)₂ 5 g, KCl 0.2 g, Mg SO_4 0.1 mg, Mn SO_4 2.5 mg, $(NH_4)_2$ SO_4 0.5 g, Ye 0.5 g and Agar 15 g with Mh 7.2. This medium was enriched with Ca_3PO_4 as phosphate source. Bacterial colonies appeared on selective medium were isolated based on their clear zone. In the early stages of this isolation process, three bacterial strains that produced widest clear zone were selected based on morphological differences.

Qualitative test of solubilizing phosphate

Pure isolates of PSB from previous screening and isolation process were inoculated on petri dishes containing solid Pikovskaya medium by spot technique. Petri dishes were then incubated at 30°C for 7-10 days. Colonies forming the clear zone more than 5.0 mm were inoculated again. Clear zone (mm) which was formed surrounding the colony was continually observed and recorded every 24 hours for 7 days.

Antagonism test

Antagonism was tested by streaking three selected bacteria from screening and isolation stage on NA (Nutrient Agar) medium on the same petri dishes and incubated at 33°C over night. Negative results if it did not form an inhibition zone when cultured simultaneously. Negative result indicated among bacteria was potential as a consortium.

Pathogenicity test

Green bean sprouts were used as the test plants for pathogenicity. Yosida medium was used for sprouts growth or growth culture. Culture stock was prepared by mixing 1000 mL solution consisting of NaH_2PO_4 40,3 g, NH_4NO_3 80 g, $CaCl_2$ 88,6 g, $FeCl_3$ 7,7 g, K_2SO_4 71,4 g, $MnCl_2$ 1,5 g, $MgSO_4$ 32,4 g, H_3BO_3 0,93 g, $(NH_4)_6Mo_7O_{24}$ 0,074 g, $ZnSO_4$ 0,035 g, and $CuSO_4$ 0,031 g. The solution for culture was prepared by mixing 30 mL of stock solution with 4 liters of distilled water and heated the solution to a mixing material, divided into reaction tubes and sterilized for 15 minutes. Green bean sprouts (2-3 pieces) were planted aseptically into tubes containing cotton and sterile Yosida medium and

then inoculated with 200 µL of pure cultures of bacteria (6 replications). All of tubes were incubated at the place with indirect sunlight. Observations were conducted every day for 5-7 days. Pathogenicity test was to observe the presence of lesions, necrosis, or abnormalities of sprouts growth comparing with control. The data of long sprouts (cm), total length of roots (cm), and fresh weights were analyzed using analysis of variance (F test) with significant value ($p < 0,05$) and if they had have significant effects they were then followed by LSD test ($p < 0,05$).

Bacterial identification

We amplified 16S rRNA Gene of bacteria that have the highest phosphate solubilizing activity and determined its sequence. Firstly, DNA isolate was extracted and its concentration was measured using PCR amplification of 16Sr RNA gene. PCR was conducted by mixing the chromosomal DNA with master mix (containing all dNTP's, Mg^{++} , BSA, primer) then initiated by the addition of taq polymerase. After amplification, PCR products were tested with agarose gel electrophoresis then purified for sequence reaction. Purified PCR products were submitted to DNA Centre Facility for sequencing. Sequencing results then are

uploaded into the GenBank online. Determination of bacterial species is needed, especially for applications in the field for security in its use.

Results and Discussion

Soil bacteria population

Estimation of soil bacterial population was conducted since we used only one medium that would enable only a few of the physiological groups of the bacterial population to develop. Estimation of soil bacterial population using NA medium showed that the highest number of population was SHR soil sample of 2.3×10^{11} CFU/mL (Table 1). This is due to the high content of organic matter in SHR soil samples (chemical soil analysis is presented in Table 2). These results are consistent with Suntoro (2003) who reported that the organic matter content of soil affects biological properties that can increase the activity and microbiological populations in the soil, especially that related to the activity of the decomposition of organic matter. SHR that had the highest number of population was also because it was a sampel from a land of forests undisturbed by human activity.

Table 1. Estimation of bacterial total population using Nutrient Agar (NA) media.

No	Soil Samples	Dilution	The average of PSB population and other bacteria
1	FHR	10^{-7}	2.3×10^{11} CFU/mL
2	SKP		3.1×10^{-9} CFU/mL
3	SJG		8.7×10^{-9} CFU/mL

Table 2. Chemical analysis of soil samples (Arfarita et al., 2016b).

Sample Code	C- Organic (%)	N-Total (%)	C/N	Organic Material (%)	P. Bray I (mg/kg)
FHR		0.19	7	2.31	4.67
SJG	1.12	0.11	10	1.94	10.56
SKP	1.31	0.15	9	2.26	27.44

SKP and SJG soil samples had a total population of bacteria of 3.1×10^9 CFU / mL and 8.7×10^9 CFU / mL. The values were lower than that of SHR because the organic matter content was lower than SHR samples. SKP and SJG were farmland and used intensively for agriculture throughout the year with tillage and chemical fertilizers input. Type of plant and soil also affect soil microbial population dynamics. However, detailed information on interactions is not yet available and still need further study. Torsvik and

Ovreas (2002) reported that total population of bacteria in the top soil is $> 10^9$ cells /g and the majority cannot be cultured. Microbial biomass that can be cultured and studied further is expected to $< 5\%$ of the population (Borneman and Triplett, 1997).

Isolation and selection of phosphate solubilizing bacteria (PSB)

Three soil samples, namely SKP (from Kendalpayak area), SJG (from Jambegede area)

and (forest soil from Junrejo area) were then performed a screening process on Pikovskaya media to get Phosphate Solubilizing Bacteria (PSB). Of 12 bacteria strain that were able to produce a clear zone were selected randomly based on morphological differences. Those

isolates were namely SPP1, SPP2, SPP3, SPP4, SPP5, SPP6, SPP6, SPP7, SPP8, SPP9, SPP10, SPP11 and SPP12. The results of screening process of PSB on solid Psikovkaya media can be seen in Figure 1.

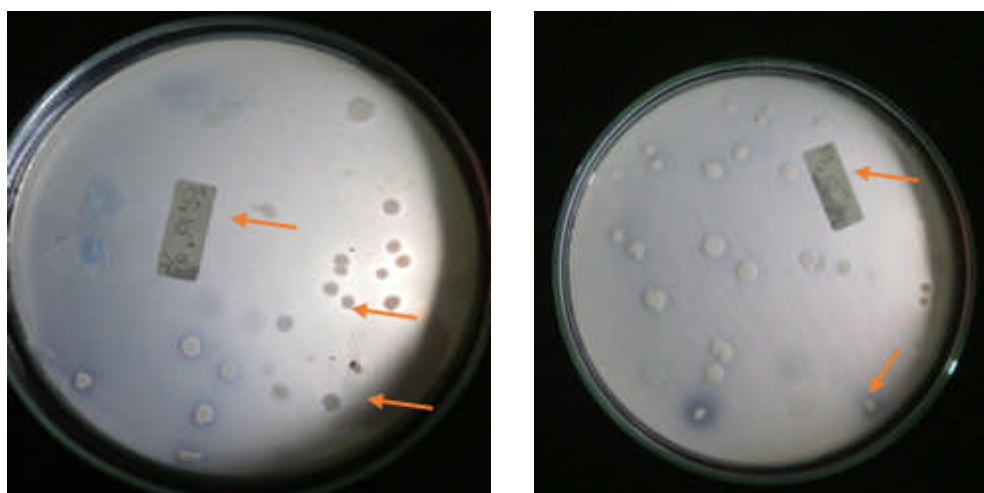


Figure 1. Screening of phosphate solubilizing bacteria on solid Pikovskaya media. Arrows indicate the colonies that are able in solubilizing phosphate by formation of clear zone.

Qualitative test of phosphate solubilizing activity

Qualitative test of 12 isolates from a screening process was conducted by the technique spot on solid Pikovskaya media. The ability of 12 isolates in solubilizing phosphate was observed by clear zone diameter. The longer of clear zone diameter indicates that the isolate is superior to other

bacterial isolates. Three superior isolates of phosphate solubilizing bacteria were then selected for further study. Figure 2 shows that strains of SPP1, SPP 2 and SPP3 have the highest phosphate solubilizing ability based on qualitative test of phosphate solubilizing activity.

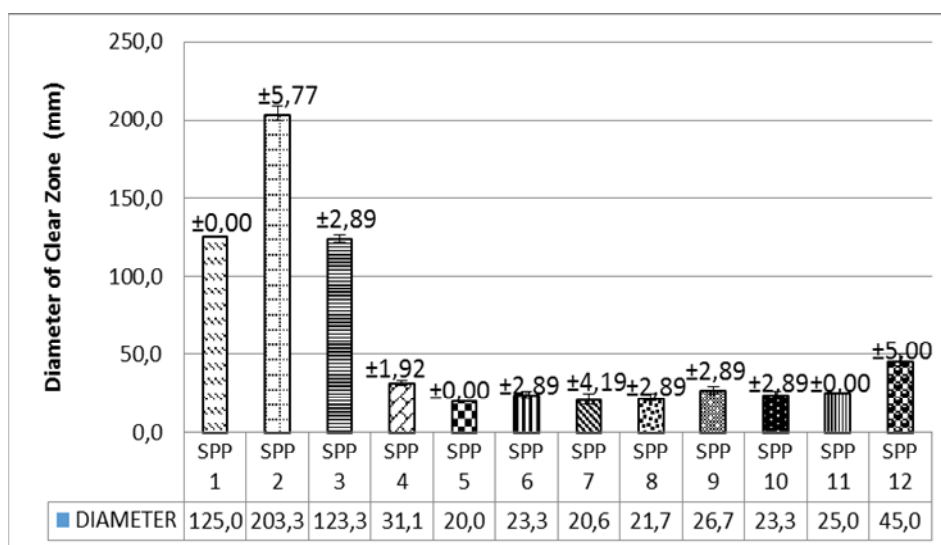
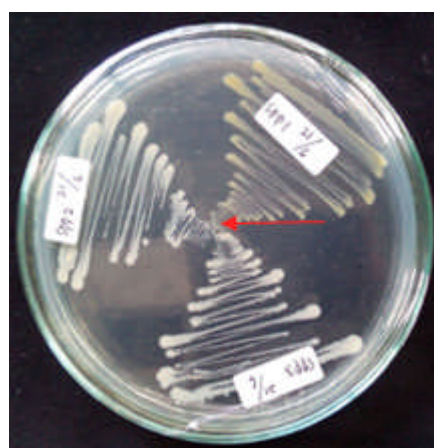


Figure 3. Diameter of clear zone formation (mm) on 12 bacterial isolates from screening process on solid Psikovkaya medium.

Phosphate solubilization activity in solid medium can be explained by a description of McGill and Cole (1981). They identified that microorganisms play an important role in all three major components of the soil P cycle (i.e. sorption–desorption, mineralization–immobilization and dissolution–precipitation). The role of soil microorganisms in term of P solubilization mechanisms, mainly include: (1) liberation of extracellular enzymes (biochemical P mineralization), (2) releasing of P during substrate degradation (biological P mineralization), and (3) release of complexing or mineral dissolving compounds e.g. organic acid anions, siderophores, protons, ions hydroxyl and CO₂.

Antagonism activity

Figure 3 (a) shows the result of antagonism test on NA media of three isolates namely SPP1, SPP2 and SPP3, which cultured simultaneously. It showed negative result of antagonist activity because it did not perform a zone of inhibition.

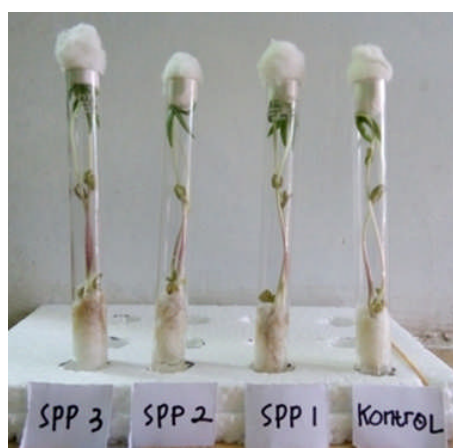


(a)

The results of antagonism test were not antagonistic so that it can be cultured together as a bacterial consortium. Antagonistic bacteria are usually found around rhizosphere that have an adverse effect on other microbes. Each microbe has its own mechanism and has more than one mechanism of inhibition. The mechanism of inhibition of biological control agents typically works by using the results of secondary metabolism, either in the form of anti-biotics, toxins, enzymes, hormones, and parasitism. The secondary metabolism is usually amino acids but other small molecules also functioning as inducers of inhibition.

Pathogenicity activities

Figure 3 (b) shows the results of pathogenicity test. Three isolates of PSB that had been selected, namely SPP1, SPP2, and SPP3 were non plant pathogenic. As shown in Figure 3(b), there are no symptoms of lesions, necrosis or abnormalities growth comparing with controls.



(b)

Figure 3. (a) Antagonism test of three selected PSB shows no inhibition after 5 days of observation, in which the end of the streak can be fused (Arrow direction). (b) Pathogenicity tests after 7 days of observation since inoculation of each PSB show no pathogenicity.

Table 4. Average of the growth of green bean sprouts after 7 days inoculation of three isolates of PSB

Treatments	Average		
	Long Sprouts (cm)	Total length Roots (cm)	Fresh weights (g)
Control	14.33 ab	37.42 b	0.96 b
SPP1	13.08 a	30.00 ab	0.85 a
SPP2	15.83 b	23.29 a	0.85 a
SPP3	14.00 a	35.00 b	0.86 a
F. Table 5%	Significant	Significant	Significant
LSD	1.68	7.96	0.09

Observations on the growth of green bean sprouts were also performed by the data of long sprouts (cm), total length of roots (cm), and fresh weights and analyzed using analysis of variance (F test) with significant value ($p < 0,05$) and if they had significant effects they were then followed by LSD test ($p < 0,05$) with 6 replicates. The results indicated that the length of the plant, total root length, and fresh weight showed a significant effect on the growth of green bean sprouts (Table 4). Average in the growth of green bean sprouts showed that there was no inhibition on growth. With a growth rate greater than control, the possibilities of selected three bacteria were to produce growth hormone. In this study, exploration of PSB potential as a biological fertilizer was also determined using 16S rRNA. Purified PCR products were submitted to DNA Centre Facility for sequencing then be uploaded into the GenBank online. Similarity test of genome sequence of strain SPP2 had 99% similarity with *Pseudomonas plecoglossicida* strain PR19. The results of this study are consistent with Kaur and Reddy (2014) on the bacteria *Pseudomonas plecoglossicida* phosphate-solvent. They isolated *Pseudomonas plecoglossicida* from organic farms then were tested for their efficacy to dissolve the phosphate rock and plant growth-promoting activities such as nitrogen fixation and production of indole acetic acid (IAA) and siderophores. They reported that *P. Plecoglossicida* dissolved phosphate and released a significant amount of phosphorus (Phosphate up to 271 mg/ ml) in the culture medium. This isolate produced IAA and siderophores, but did not manage to fix nitrogen, which was determined by acetylene reduction. Two-year field study was conducted to the effectiveness of these bacteria on the growth and yield of corn and wheat crops grown on organic farms with and without rock phosphate. This study showed that *Pseudomonas plecoglossicida* and giving phosphate rock plays an important role in enhancing the productivity of crops in organic farming. In addition to *Pseudomonas*, it has been reported that other bacteria observed as P-solubilizers including *Klebsiella*, *Enterobacter*, and *Pantoea*, (Chung et al., 2005), *Xanthomonas* (De Freitas et al., 1997), *Azotobacter* (Kumar et al., 2001), *Arthrobacter*, *Chryseobacterium*, *Delftia* sp., *Gordonia*, *Phyllobacterium*, *Rhodococcus*, *Serratia*, (Wani et al., 2005; Chen et al., 2006), *Vibrio proteolyticus*, *Xanthobacter agilis* (Vazquez et al., 2000). Zaidi et al. (2009) reported that symbiotic nitrogenous *rhizobia*, which fix atmospheric nitrogen into ammonia and export the fixed nitrogen to the host plants, also shown phosphate solubilizing activity. For

example, *Rhizobium* species nodulating *Crotalaria* species (Sridevi et al., 2007) and *Rhizobium leguminosarum* bv. *Trifolii* (Abril et al., 2007), improved plant P-nutrition by mobilizing inorganic and organic P. Various phosphate solubilizing bacteria have also been isolated from extreme environments for example the halophilic bacteria *Kushneria sinocarni* isolated from the sediment of Daqiao slattern on the eastern coast of China, which probably useful in salt affected agricultural soils (Zhu et al., 2011).

Conclusion

The total population of soil bacteria from each sample was different. Soil sample from Junrejo (Forest Area) had a total bacteria population of 2.3×10^{11} CFU/mL. While soil samples from area Kendal Payak and Jambe Gede were much lower than that of forest land, with total population of 3.1×10^9 CFU/mL and 8.7×10^9 CFU/mL, respectively. Three potential bacteria that enabled to dissolve phosphate had been selected, namely SPP1, SPP2, and SPP3. Pathogenicity test did not show that these three strains were pathogen against green bean sprouts. Antagonism tests on three selected isolates showed no inhibitory zone occurred simultaneously when cultured on NA. All three isolates have possibility in producing growth hormone, after germination was observed in green bean sprouts. SPP2 then selected for next further study based on the highest activity of phosphate solubilizing. Similarity test of genome sequence of strain SPP2 had 99% similarity with *Pseudomonas plecoglossicida* strain PR19. *Pseudomonas plecoglossicida* has never been reported to be pathogenic to plants and even some studies showing its effectiveness in dissolving phosphate. Bacteria selected in the study has potential to be developed as an agen of biofertilizer.

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