ISOLATION AND CHARACTERIZATION OFPHOSPHATE SOLUBILIZING BACTERIA FROM RHIZOSPHEREOF PINNEAPLE PLANTATION IN LAMPUNG, INDONESIA

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Abstract

Cultivation of pineapple (*Ananascomosus*) on a large scale plantation often use several fertilizers in high dosage. Utilization of rhizosphere microbes such as phosphate solubilizing rhizobacteria (rPSB) was expected to help reducing the dosage of inorganic P fertilizer application due to its capability in dissolving phosphate fixed by Al and Fe. By those, P become more available for plants. This study aims to evaluate the activity of rPSB isolated from pineapple plantation, both in fields/blocks of low and high pineappleproduction, in Lampung province. rBPF was isolated from pineapple plantation blocks with low (R) and high (T) production levels, as well as in the vegetative (V) and generative (G) stages of plant, with 5 replications.The results showed that the PSB population was around 10^3 CFU/g. Morphological characteristics of PSB isolateswere mostly white, yellowish, gram-positive, and rod-shaped. The optimum pH for average growth was at pH 5.0, while the optimum temperature was at 30°C. The five best isolates from each block were reconfirmed their phosphate solubility ability based on the phosphate solubility index (PSI). The best PSI of isolates in each block were TG3 (0.80), RV5 (1.05), TV1 (1.10), and RG2 (1.20). The increase in the amount of BPF from TV isolates to RV and TG to RG is in line with the increase in available P (Br-P) soil levels from 25.57 to 40.68 and from 23.83 to 25.25 ppm. This indicatesincreasing of PSB activity with the available P level of the soil.Based on 16S rRNA identification, isolate RG2 was similar 99.69 % to *Bacillus altitudinis*

Keywords: *Bacillus altitudinis*, P-Bray, molecular identification, phosphate solubility index.

Introduction

Activities of pineapple cultivation that have been carried out by plantations for many years have resulted in reducing quality of soil fertility. This can be caused by the use of fertilizers and pesticides in each pineapple growth cycle. Several inorganic fertilizers -such as Urea, K_2SO_4, $CaCl_2$, DAP, $MgSO_4$, Dolomite, and borax- were used in pineapple cultivation processes by many

plantations. The use of those fertilizers have caused soil hardening and reducing the soil fertility.According to previous research conducted by (Sherman and Brye 2019), the physical properties of ultisol soils in the pineapple plantations are mostly in the low and very low qualities, so that some plantation blocks have low production levels. The difference in production levels caused by declining soil quality is one of the problem occurs, so it is necessary to study the presence of microbial species exist around the rhizosphere. In addition, it is also a need to study them at the different growth stages of plant. It is because according to a research conducted by (Sakimin*et al.*,2017), the need of fertilizer dosage in each phase of pineapple growth was different.

The difference in dosage of fertilizer applied in the vegetative and generative phases will also affect the soil conditions.One of the most frequent efforts is adding potential phosphate solubilizing bacteria (PSB) as biological fertilizer (biofertilizer), e.g. *Pseudomonas* sp., *Bacillus* sp., *B. megaterium*, and *Chromobacterium*sp. are PSB with high ability as biofertilizer, making P available for plants (Widawati and Suliasih 2006). Fitriatin*et al.*, (2008)utilized PSB as biofertilizer, resulting in increasing phosphatase enzyme activity in soil because the biofertilizer is capable of increasing plant growth. PSB are soil microbe groups often utilized to improve soil that was previously in bad condition due to fertilizer application (Sharma *et al.*,2013). More soil biological, physical, and chemical reactions are in rhizosphere because activities in rhizosphere are supported by nutrients in their soluble exudate forms secreted by plants, such as carbohydrate, amino acid, low molecular weight organic acid,and other photosynthates(Egamberdieva*et al.*,2010).

Rhizosphere's ecology is driving factors, combining soil matrix's physical factors, rhizodeposit (root exudate), proton (H+), gas, and root's role in absorbing water and nutrients, that are spatially and temporally well-distributed, allowing root areas become sites where soil microbes, plant roots, and soil components interact (Gregory 2006,Richter*et al.*, 2007). The purpose of this study is to determine the amount of rBPF contained in the pineapple rhizosphere with different production levels, isolate BPF that has a high solubility index, and determine the characteristics of BPF isolated from the pineapple rhizosphere.

Materials and Methods

Time and Location

The present study was carried out in January to July 2018 using descriptive method by obtaining soil samples from soil rhizosphere in 4 blocks of PT Great Giant Pineapple (GGP), Lampung Tengah, Lampung Province, and analyzing the samples at Laboratory of Soil Biology, Biotechnology, Faculty of Agriculture,Bogor Agricultural University.

Soil Sampling

Random samplingswere carried out in 4 locations with 5 replications each, totaling 20 samples. Soil samples of 100 gr each were obtained from around pineapple root system areas at 0-15 cm deep (Figure 1) and put in labelled plastic

bags for biological analysis. The 4 sampling locations were Block RV (4°47' S, 105°18' E), RG (4°48' S, 105°15' E), TV (4°49' S, 105°11' E), and TG (4°47' S, 105°12' E).

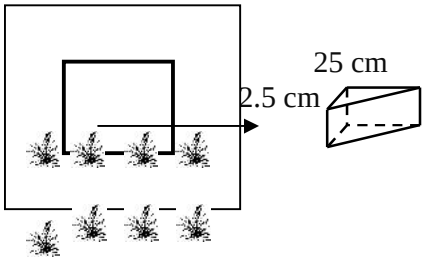

Fig. 1: Soil sampling from pineapple rhizosphere

Analysis P

Determination of the P element content was carried out by the wet ashing and P Bray methods. Wet ashing is done by using a mixture of $HClO_4$ and HNO_3 solutions

PSB Isolation and P Solubilizing Test

A total of 5 g soil was put in 45 ml sterile physiological solution and shaken for 30 minutes in shaker. 1 ml extract was then put in test tube with 9 ml sterile physiological solution and shaken to homogeneous. 1 ml extract was then put in the next test tube and so on until 10^{-1}-10^{-6} serial dilutions were obtained. 1 ml extract from each 10^{-3}-10^{-6} serial dilution was put in sterile petri dish and Pikovskayaselective media was poured in prior to incubation for 3-5 days at ±25^{o}C. Colonies with clear zone were observed and counted. Desired colonies were then purified using quadrant streak method onPikovskaya medium to obtain single colony. Isolates obtained were stored as stocks for further test (Suliasih 2012, Ulfiyati*et al.* 2015). Phosphate solubilizing ability of the PSB isolate was tested to obtain phosphate solubilizing index (SI)usingequation below:

$$SI = \frac{\text{Clear Zone diameter} - \text{Colony diameter}}{\text{Colony diameter}}$$

PSB Characterization

PSB isolates which had rapid growth and high SI were characterized against their colony and cell morphology. Gram staining was used to stain bacteria cells and group them into Gram-positive and Gram-negative.

Biochemical Test

1 loopmicrobe producing sample bacteria was inoculated on biochemical test media (glucose, fructose, sucrose, lactose, and galactose) prior to incubation for 24 hours at room temperature 25^{o}C. Observation was carried out on color changing of media and gas producing at Durham tube.

Influence of pH on Bacterial Growth

Sample PSB candidates were taken from ultisolsoils with pH 4.5-5.5. It is necessary to understand the optimum pH for the growth. Samples obtained were streakedonto Nutrient Agar (NA) media at pH 5 and 7 prior to incubation for 24 hours at room temperature 25^{o}C. After incubation, size of colonies growing on the media was observed.

Influence of Temperature on Bacterial Growth

Bacterial samples obtained were streaked onto NA media prior to incubation at 30^{0}C and 40^{o}C. After incubation, size of growing colonies on the media was observed.

Polymerase Chain Reaction (PCR)

PCR process begins with the making of 75 µl PCR mixture reaction withcomposition: 7.50 µlof 10x PCR buffer, 2.25 µldNTP10 mM, 1.50 µl MgSO450 mM, 0.75 µl primary F 10 µM, 0.75 µl primer R10 µM, 1.00 µl DNA template, 1.00 µlTaq DNA polymerase, and 60.25 µl sterile aquabidest. PCR was running for 30 cycles with the following conditions: denaturation of the initial cycle or pre-denaturation at 94ºC for 5 minutes, followed by denaturation for the next cycle at 94ºC for 30 seconds, primary annealing for 30 seconds at 50ºC, and polymerizationfor 2 minutes at 72°C.In the last cycle, ie 30th cycle, polymerization was extended for 7 minutes. Finally, temperature was reduced to 4ºC to stop PCR reaction. PCR product was visualized using 1.0% agarose gel electrophoresis in 0.5x TAE buffer for ± 45 minutes, with 110 Volt in voltage.

DNA Purification of PCR Results

Purification of PCR results was carried out through cutting agarose gels containing DNA bands resulting from PCR. The gel cut was weighed as much as 100 mg then put in eppendorf 1.5 ml and 200 ml of NT buffer was added. The sample was incubated for 5-10 minutes at 50 ° C then firmlyorted. The sample was placed in the NucleoSpin Extract II column which had previously been placed in a collection tube (2ml) then centrifuged. Then µl buffer NT3 (washing buffer) was added to the NucleoSpin Extract II column and centrifuged at 11,000 x g for 1 minute until all buffers moved to the collection tube.

Analysis of 16S rRNA NCBI BLS DNA coding data

The sequencing of pure DNA (sequencing) is done by sending purified DNA from the product to the University of Lampung Biomolecular Laboratory. Bioedit software (Tom HALL, Ibis Theraupetics) is used for analysis of rough data from the sequencing results and then the data that has been processed is matched with the data in the gene bank http://blast.ncbi.nlm.nih.gov/ which will show the kinship of the species in a way genetic (Philogenetic Tree).

Results and Discussion

Available Soil P (P-Bray)

The availability of P in the pineapple rhizosphere in almost all locations shows that the level of P available is relatively very high (Table 1), the high level of available P can be caused by the activity of microorganisms in the pineapple rhizosphere

Tabel 1 :P available at the soil sampling location

Location	**Mean of P available**(ppm)
RV	40.68(VH)
TV	27.57(VH)
RG	25.25(VH)
TG	23.83 (VH)

Note: based on Sulaeman*et al*. (2012), VH: Very high

The presence of phosphorus (P) plays an important role in process of energy storage and transferwithin the body of the plant. Inadequacy of P cause plants to not grow optimally. In this research, P content in pineapple rhizosphere in almost all locations showed that the level of available P was relatively very high (Table 1). High level of this available P could be caused by several factors, such as activity of microorganisms and content of soil organic matter around the rhizosphere. In some studies (Islam*et.,* 2014), it was proven that the addition of compost as an organic material had a positive influence on the solubility of phosphate in the soil so that it could increase the availability of P. Several soil microbial groups also play important role in dissolution of various soil Pso that bounded-P gradually releases into dissolved-P. Micromonospora species, *Pseudomonas* sp., have a significant contribution to the process of P mineralization in the rhizosphere of plants(Alori *et al.,* 2017).

PSB Isolated from Pineapple Rhizosphere

The role of microorganisms in the cycle of nutrient availability, making microorganisms come into a determinant player in terms of soil fertility and health. Changes in soil body conditions can also be seen from population dynamics and bacterial activity. According to this research, PSB populations isolated from 4 locations were around 10^3CFU/g, where the highest population was from RG location and the lowest was from TV location (Figure 2).

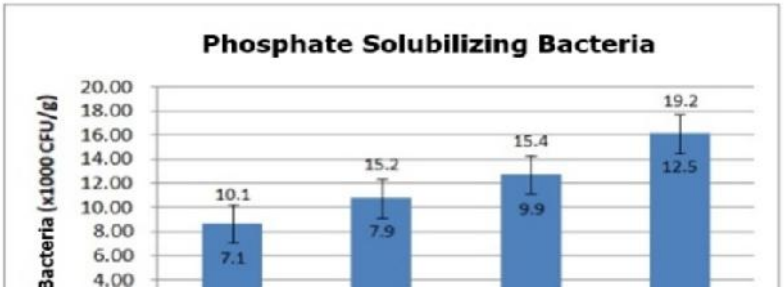

Fig. 2: Total PSB from different locations

The range of smallest solubility index was 0.65 (isolate TV5) to 1.2 (isolate RG2) (Table 2). Most BPF isolates were able to ferment glucose, galactose, fructose, and only few were able to ferment lactose. Those isolates can grow well at temperatures of 30^{o}C and pH 5. Isolate RG2 was Gram-positive bacteria and have greatest IKF among others. Isolate RG2 grew on Pikovskaya medium surrounded by clear zone around their colonies (Figure 3) which shows the ability of isolates to dissolve phosphate contained in Pikovskaya media. Isolate RG2 have not ability to ferment lactose, and can grow well at pH 5 and 7 (Table 3).

Fig. 3: Clear zone around colony of phosphate solubilizing bacteriaon Pikovskaya media

Table 2: Phosphate SI

Isolate	Phosphate SI	Isolate	Phosphate SI
TV1	1.1	RV1	0.7
TV2	0.75	RV2	0.85
TV3	0.95	RV3	1.0
TV4	1.1	RV4	0.75
TV5	0.65	RV5	1.05
Average	0.9	Average	0.76
TG1	0.75	RG1	0.85
TG2	0.75	RG2	1.2
TG3	0.8	RG3	1.0
TG4	0.75	RG4	0.80
TG5	0.75	RG5	0.80
Average	0.74	Average	0.93

Table 3: Bacterial characterization based on biochemical properties, pH, temperature, and Gram staining

Isolate Name	Biochemical Test					pH		Temperature (^{0}C)		Gram Staining
	Glu	Gal	Fru	Suc	Lac	5	7	30	40	
TV1	+++	+	+++	++	-	+++	+	+++	+	(-)
TV2	+++	++	++	++	+	+++	++	+++	+	(-)
TV3	+++	++	+++	++	++	+++	+	+++	+	(+)
TV4	+++	++	+++	++	-	+++	++	+++	+	(-)
TV5	+++	++	+++	++	-	+++	++	+++	+	(-)
TG1	+++	++	++	++	-	+++	++	+++	+	(+)
TG2	+++	++	+++	++	-	+++	+	+++	+	(-)
TG3	+++	+	+++	++	-	+++	+	+++	++	(+)
TG4	+++	++	+++	++	-	+++	++	+++	+	(-)
TG5	+++	+	+++	++	-	+++	++	+++	++	(-)
RV1	+++	++	+++	++	+	+++	++	+++	++	(-)
RV2	+++	++	++	++	-	+++	++	+++	+	(+)
RV3	+++	++	++	++	+	+++	++	+++	+	(-)
RV4	+++	++	+++	++	-	+++	++	+++	++	(+)
RV5	+++	++	+++	++	-	+++	++	+++	+	(+)
RG1	+++	++	+++	++	-	+++	++	+++	++	(-)
RG2	+++	++	+++	++	-	+++	++	+++	++	(+)
RG3	+++	+	+++	++	-	+++	+	+++	+	(-)
RG4	+++	++	++	++	-	+++	++	+++	+	(+)
RG5	+++	++	++	++	-	+++	++	+++	+	(-)

Note:
+ = Slightly grow, ++ = Moderately grow, partial fermentation
+++ = Abundantly grow, full fermentation
Glu = glucose, Fru = fructose, Gal = galactose, Suc = sucrose, Lac = lactose

The available P content affects the life of phosphate solubilizing bacteria in pineapple rhizosphere.According to(de Souza *et al.*,2015) the activity and number of soil bacteria increase with the proximity of soil bacteria from plant roots.The relationship between population of phosphate solubilizing bacteria and the content of soil available P bind the soil content.Phosphate solubilizing bacteria have a very large ability as a biofertilizer in dissolving phosphate fixed in the soil by Fe, Al, Ca, and Mg, so that these elements can be dissolved by bacteria and then become available for plants.Increasing of the total number of potential microbes from TV isolates to RV and TG to RG (Figure 2) is in line with the increasing of soil available P (P-Bray) levels from 25.57 to 40.68 and from 23.83 to 25.25 ppm (Table 2).This indicates that increasing of rBPF activity would increase available P levels in soil.Soil microbes such as *Pseudomonas* sp. and *Bacillus* sp. can release organic acids such as formic acid, acetic acid, and lactate which can dissolve difficult-to-dissolve forms(Susilowati and Syekhfani 2014,Saeid *et al.*,2018).

Organic acids released by these bacteria can form chelate (stable complex) with P-binding cations, such as Al^{3+} and Fe^{3+}, in natural soils. The chelate can decrease the reactivity of these ions, causing effectively phosphate dissolution so that it can be absorbed by plants.Phosphate solubilizing bacteria (BPF) in addition to increase the availability of soil P is also a nutrient pool (mainly P) so that it is a driving force for soil ecosystems, controlling the rate of change in nutrient forms especially P in the form of dissolved-P, insoluble-P, or absorbed-P, and other forms of organic P. Phosphate solubilizing bacteria through enzymatic process (Baldotto *et al.*,2014)and the release of organic acids (such as malate, citrate and oxalate) produced by BPF, play important role in releasing of embedded-P (Al-P, Fe-P, and Ca-P) into the form of P that available to plants and soil biota, neutralizing the influence of toxic metals, and weathering the soil minerals.

The total population of bacteria found in the soil is not the same, this depends on soil type and ability to uptake nutrients in soil. (Taha *et al.*,1969)stated that soil chemical and physical factors such as vegetation, plant rotation, and environmental condition was highly influence of soil microbe population, including PSB. PSB population compositions and activities will be diverse due to different soil physical-chemical properties, climate, and vegetation(Liu*et al.*, 2013).

According to Islam*et al.*,(2010), the number of microbe population around soil depends on the microbe's metabolic sensitivity against the surrounding conditions, e.g. humidity, light intensity, and soil temperature. It means PSB population in soil is also influenced by temperature, considering temperature's role in cell physiological reaction, and environment's physical-chemical characteristics including soil volume, pressure, oxidation-reduction potential, diffusion, brown movement, viscosity, surface tension, and water structure(Shanware, Kalkar, & Trivedi, 2014). Other study indicated that there is a seasonal change in rhizosphere microbe population in line with root growth(Semenov *et al.*, 1999), making newly growing root, mature root, old root, and decaying root have different community compositions. Bacterial community obtains nutrients released by young roots in root hair area.

Bacteria's different SI is due to bacteria's different ability. P solubilizing process is presumably due to organic acids production. According to Khan *et al.*,(2007), the main mechanism releasing P is due to organic acids productionby PSB. Organic acid production decreases pH, and P is released through proton substitution. Organic acids produced then react with Ca^{2+}, Fe^{3+}, and Al $^{3+}$ions that bind phosphate into stable form and phosphates are released in forms available for plants(Shen *et al.*, 2011).Study by Setiawati and Mihardja (2008)reported that there are several microbe mechanisms in solubilizing phosphate, e.g. orthophosphate anion competition, pH change due to organic acids productions, metal binding to form organic metal, and chelation by organic ligand.

Morphological characteristics of the 12 PSB isolates (Table 2) revealed that most of the PSB colonies wereround-shaped, white, yellow, Gram-positive (5 isolates), and Gram-negative (7 isolates). Physiological characteristics of the isolated PSB revealed that most PSB were capable of fermenting carbohydrates in glucose, fructose, galactose, and sucrose forms, where only isolate RV1 and TV3 were capable of reducing lactose. Optimum pH for bacterial growth was at pH 5 and 7, although isolate RG5 and TV1 only grew slightly at pH 7. According to (Widawati and Suliasih 2006), maximum pH for phosphate availability in soil is 6.5, while PSB is still capable of solubilizing phosphate at pH below 5.5. PSB isolates were obtained from pineapple rhizosphere at 0-15 cm deep where the temperature at the soil layer fluctuated from 25 to 33 ^{0}C at noon, especially during dry season. This is due to the soil temperature at the layer is highly influenced by the temperature of the environment above (Balai Penelitian Tanah 2005).

Based on 16S rRNA identification, isolate RG2 with the highest SI was similarity 99,69 %to *Bacillusaltitudinis*(Figure 4.).

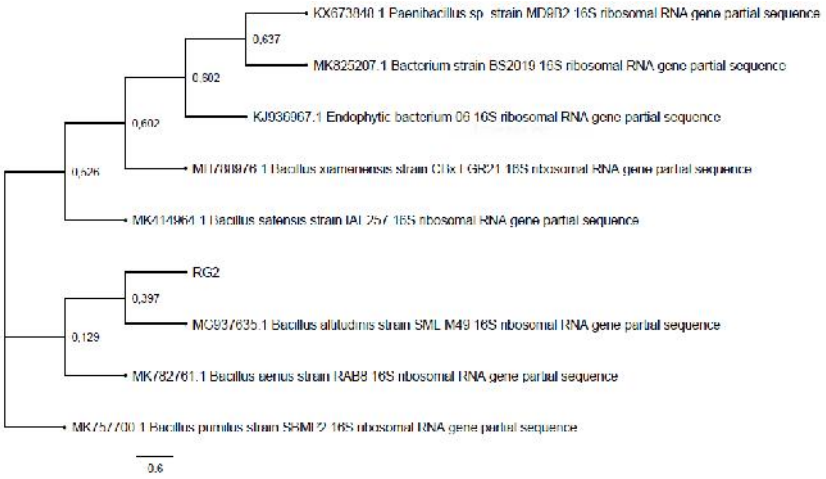

Fig. 4: Filogenetic tree of isolate RG2

Conclusion

A total of 20 potential PSB isolates were successfully obtained from pineapple rhizosphere. P dissolving test revealed that isolate TV5 had the lowest SI (0,65) and isolate RG2 had the highest (1.2). Morphological characteristics of PSB isolates were mostly white, yellowish, gram-positive, and rod-shaped. The optimum pH for average growth was at pH 5.0, while the optimum temperature was at 30°C. Based on 16S rRNA identification, isolate RG2 with the highest SI was 99,69 % similar to *Bacillusaltitudinis.*The mean PSI isolates of RV, RG, TV, TG were 0.76, 0.93, 0.6 and 0.74, respectively. The increase in the number of rBPFs from TV isolates to RV and TG to RG is in line with the increase in available P-Bray (P-Bray) soil levels from 25.57 to 40.68 and from 23.83 to 25.25 ppm. This indicates that increasing rPSB activity increases P levels of available soil.

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