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Isolation, Characterization, and Molecular Identification of Phosphate Solubilizing Bacteria from Several Tropical Soils

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ABSTRACT

The objectives of the research were: (i) to isolate and characterize of phosphate solubilizing bacteria (PSB) and (ii) to identify PSB based on molecular amplification of 16S rRNA gene. Soil samples were collected from rhizosphere in Bogor, West Nusa Tenggara, and East Nusa Tenggara. Several stages in this research were: (i) isolation PSB in Pikovskaya agar, (ii) morphological and biochemical characterization of PSB, (iii) measurement of phosphatase enzymes, and (iv) measurement of secreting indole acetic acid phytohormone. As many as 29 isolates of PSB have been collected and three isolates of them, namely: P 3.5 (East Nusa Tenggara), P 6.2 (West Nusa Tenggara), and P 10.1 (Citeureup, West Java) were chosen for further study. There were many characteristics of isolate P 10.1: (i) it had capable to solubilize P with the value of highest solubilization index (1.80), (ii) it had the highest phosphatase enzyme (120.40 mg kg^{-1}), and (iii) it had the highest pH decrease at each observation for six days. Isolates P 3.5 and P 10.1 were the Gram-negative bacteria with coccus shapes and isolate P 6.2 was a Gram-negative bacteria with bacillus shape. Deoxiribonucleat Acid (DNA) amplification of these bacteria employing 16S rRNA primers generated the 1,300bp-PCR product. The results of the analysis of 16S rRNA gene sequences showed that isolates P 3.5 and P 10.1 has 98% similarity with *Gluconacetobacter* sp. strains Rg1-MS-CO and isolate P 6.2 has 97% similarity with *Enterobacter* sp. pp9c strains.

Keywords: 16S rRNA, indole acetic acid, isolation, phosphatase enzymes, phosphate solubilizing bacteria

INTRODUCTION

Phosphor (P) is one of the macro element which is very important for plant growth. However, content of P in plant is lower than nitrogen (N), Potassium (K) and Calsium (Ca) (Havlin *et al*. 2005). Phosphate fertilizing often do not eficient because phosphate is bonded in unavailable form, so it is not available to be uptake by plant. The main problem in phosphate fertilizing on farmland is low in efficiency, because only 10-30% that can use by plant. This happenend because bounding process or fixation of P is high enough by soil. To increase efficiency of phosphate fertilizing, nowdays it had improvement to use phosphate solubilizing microorganism, such as phosphate solubilizing bacteria (PSB).

Research by Olievera *et al*. (2009) showed that PSB produced the greatest solubilization in medium containing tricalcium phosphate [$(Ca_3(PO_4)_2$]. This is suitable with research by Coutinho *et al*. (2012) which showed that 90% of PSB isolates had potential for solubilization of *simple superphosphate* (SSP) and *mono-ammonium phosphate* (MAP) on the seventh day of evaluation, with average values of respectively 23% and 22% higher than control. Park *et al*. (2011) explained that inoculation of PSB with rock phosphate (RP) in soil can be used as an alternative technique to soluble P compounds. Research by Yang *et al*. (2012) showed that PSB had different abilities to solubilize tricalsium phosphate, with the concentrations of solubilized P in bacterial cultures varied from 33.48 to 69.63 mg L^{-1}. According to research by Montanez *et al*. (2012) some genera of PSB are *Rhanella, Pantoea, Rhizobium, Pseudomona, Herbaspirillum, Enterobacter, Brevundimonas* and *Burkholderia*. All strains produced IAA in vitro but only *P. fluorescens* (EMA68) produced siderophores. Phosphate solubilizing bacteria is useful and it can be useful as an alternative that very potential to improve available-P element in the soil.

The objectives of this research were to isolate, characterize, and indentifiy PSB from some locations in Bogor, West Nusa Tenggara (WNT), and East Nusa Tenggara (ENT), that had high ability to solubilize P element and to determine kind of bacteria through molecular identification according to amplification of gen 16S rRNA.

MATERIALS AND METHODS

Soil Sampling and Media

Tropical soil samples were collected from Experiment Station Bogor Agriculture University, Cikabayan (Bogor, West Java), Citeureup (Bogor, West Java), West Nusa Tenggara (WNT), and East Nusa Tenggara (ENT), Indonesia

Isolation and purification of PSB strains were carried out by Pikovskaya Medium, (with 2 different sources of P, $Ca_3(PO_4)_2$ or tricalsium phosphate and $Ca_5(PO_4)_5OH$ or hidroxy apatit), modified universal buffer (MUB) 1×, 0.5 *N* NaOH, 0.115 *M* p-nitro phenil phosphate (p-NPP), 1 mg mL^{-1} p-nitrophenol (p-NP), standar solution *indole acetic acid* (IAA), Salkowski reagent, cristal violet, iodium safranin, alcohol 95%, sterile distilled water.

Isolation of PSB from Rhizosphere Soils

A total of 10 grams of soil from each soil sample was added into a tube containing 90 mL Pikovskaya liquid medium at pH 6.8, then it was made serial dilutions up to 10^{-4}. A total of 50 mL of 10^{-3} and 10^{-4} dilution was poured over the surface of solid medium in a sterile Pikovskaya medium, then spread evenly using the spreader bars, and incubated for 2-3 days at room temperature. The presence of PSB growth was marked by a clear zone of light-colored or clear zone around the colonies that showed a dissolution of phosphate.

Characterization and Ability of PSB in Dissolving P

After PSB that was isolated from some soil samples, they were characterized as morphology by observation on colony of PSB which included shape, edge, elevation, and colour as suitable with Hadioetomo procedures (1993). On other hand, testing capabilities of PSB in dissolving P in solid medium was also conducted by measurement the diameter of Pikovskaya clear zone and dissolution index (DI) of phosphate.

$$DI = \frac{\text{Diameter of colony} - \text{light zone}}{\text{Diameter of colony}}$$

Observations made during the 7 days of incubation by observing the shape, edge, elevation, and color as well as measuring the diameter of clear zone and DI of phosphate PSB colonies that produced clear zone around the colony.

Phosphatase Enzyme Measurement

Phosphatase enzyme measurements were carried out in accordance with procedures of Tabatabai and Bremner (1969). A total of 10 mL PSB cell culture was grown for 3 days at room temperature in a liquid Pikovskaya medium (with two sources of P that were $Ca_3(PO_4)_2$ and $Ca_5(PO_4)_3OH$) and was centrifuged (10,000 rpm, 15 min) to separate the bacterial colonies from medium. The supernatant was transferred into a clean and sterile test tube for 1 mL, then it was added 4 mL of Modified Universal Buffer (MUB) 1×, and 1 mL of 0115 *M* p-nitro phenyl phosphate (p-NPP), and incubated for 60 min at in a waterbath at 37 ºC. After incubation for 60 minutes, a 5 mL of 0.5 *N* NaOH was added to stop the reaction. The solution was turned yellow, indicating that the bacterium produced the enzyme phosphatase. Absorbance measurements were performed at a wavelength (ë) = 410 nm using a Hitachi 150-20 Spectrophotometer type.

Hypergrowing Indole Acetic Acid of PSB

Indole Acetic Acid (IAA) measurements were carried out in accordance to procedures of Gordon and Weber (1993). A total of 10 ml PSB cell culture was grown for 3 days at room temperature in a liquid medium Pikovskaya (with two sources of P), then it was centrifuged (10,000 rpm, 15 min) to separate the colonies from the medium. The supernatant was transferred into a clean and sterile test tube of 2 mL, and to 2 mL Salkowski reagent (ratio 1:1). Salkowski reagent mixture and the supernatant was incubated for 60 minute at room temperature. The solutions were turned pink, this was an indication of the content of IAA. The solution absorbance was measured at a wavelength (λ) = 530 nm using a Hitachi 150-20 Spectrophotometer type.

Population Growth, pH, and Content of Phosphatese Enzyme of PSB

Measurement of pH changes in the liquid Pikovskaya medium of PSB isolates were carried out using a pH meter. A population growth PSB was measured by making serial dilutions of 10^{-5}, 10^{-6}, and 10^{-7}. Measurement of phosphatase enzyme content was similar to the previous. All measurement was carried out for 7 days of incubation, starting from day 0 to day 6.

Gram Staining the PSB

Gram staining test were done according to Hadioetomo (1993) in which Gram-positive bacteria appeared purple, while Gram-negative bacteria appeared pink.

Bacteria Genomic DNA Isolation

Isolation of total genomic DNA used a modification of the method of Lazo *et al*. (1987).

DNA Electrophoresis Process

A total of 2 ml of each DNA was mixed with 3 ml loading buffer as ballast. Suspension of DNA with loading buffer solution was injected into the wells on gel electrophoresis. After all wells were filled, the electrophoresis power supply was switched with a voltage by 110 V for ± 45 minutes. Gel electrophoresis results were appointed and subsequently immersed in a solution of ethidium bromide (EtBr) for 10 minutes for staining (staining) and destaining in distilled water for 5 minutes. Further, DNA can be viewed and photographed using UV Transilluminator device equipped with a digital camera.

Polymerase Chain Reaction Analysis

Primers were used in the polymerase chain reaction (PCR) process, namely the F-63 primer (5'-CAGGCCTAACACATGCAAG TC) and R-1387 primer (5'-GGGCGGCGTGTA CAAGGC) (Marchesi 1998). The process was begun by the manufacturing protocols in which a mixture of PCR reaction components for PCR in 75 ml with the following composition: 7.50 ml 10× PCR buffer, 2.25 ml 10 m*M* dNTP, 50 m*M* MgSO4 1:50 mL, 0.75 ml 10 ìM primer F, 0.75 ml 10 ìM primer R, 1:00 ml template DNA, 1.00 mL Taq DNA polymerase, and 60.25 mL of sterile aquabidest.

Total running of 30 cycles of PCR were performed with the following conditions: the initial cycle of denaturation or pre-denaturation 94 ºC for 5 minute, followed by denaturation for the next cycle at 94 ºC for 30 seconds. Annealing was performed for 30 seconds at 50 ºC. Polymerization carried out for 2 minutes at 72 ºC and the last cycle, the cycle-30 was made an extension for 7 minute polymerization time. Recently, it was performed to a temperature drop of 4 ºC to stop the PCR reaction. PCR reaction were perfomed in Swift™ Maxi Thermal Cycler Blocks-"ESCO". PCR products were visualized by using 1.0% agarose gel electrophoresis in 0.5× TAE buffer with a voltage of 110 volts for ± 45 minutes performed on Mupid-2 plus. PCR results were checked by a Scope UV Transilluminator (Destkop Gel Image-SCOPE 21).

DNA Sequencing

Polymerase Chain Reaction products were sequenced using an Automated DNA Sequencer tool PRISM ABI 377 (Perkin Elmer Biosystem, USA). Cycle DNA sequencing kit template was performed using BigDye ® Ready Reaction Mix (Perkin Elmer Biosystem, USA). DNA sequences obtained were compared to sequences in the database the European Bioinformatics Institute (EBI) using WU-BLASTN 2.0 tools on the site HYPERLINK "http://www.ebi.ac.uk"http://www.ebi.ac.uk.

RESULTS AND DISCUSSION

Isolation of PSB

Phosphate solubilizing bacteria which grows in dense medium will dissolve P Pikovskaya which is characterized by clear or light-colored zone surrounding the bacterial colonies. This is due to the dissolution of phosphate from $Ca_3(PO_4)_2$ which is contained in the medium. A total of 29 isolates of the PSB which produced clear zone was then purified on a solid medium Pikovskaya (Table 1).

Characteristics and Ability of PSB in dissolving P

PSB isolates were observed its colony morphology characteristics included the shape, edge, elevation, and the color of bacterial colonies according to the procedure Hadioetomo (1993), as well as the measurement of phosphate dissolution index (DI). Observations and measurements are shown in Table 1.

Based on DI generated it could be seen that the ability of PSB to dissolve P was varied. This suitable with research by Liu *et al*. (2011) which showed that 15 PSB strains exhibited inorganic P-solubilizing abilities ranging between 376.62 and 669.56 mg L^{-1}. According to Rachmiati (1995), a qualitatively clear zone area allegedly showed the size of the ability of bacteria dissolving P from insoluble phosphate.

The Ability of PSB in Produce Phosphatase Enzyme, IAA and Gram Staining

The ability of the PSB in the enzyme phosphatase and IAA production as well as the Gram staining results are shown in Table 2.

Phosphatase enzyme is an important enzyme complex in the soil is functioned to change a phosphate which is bonded in organic compounds into a form available to plants. Table 2 shows that PSB isolates that produced the highest phosphatase enzyme in phosphate source of $Ca_3(PO_4)_2$ was P 3.3 isolates with phosphatase enzyme content of 98.22 mg kg^{-1}.

Table 1. Morphological characteristics and the ability of PSB to dissolve P on Solid Pikovskaya medium during the 7 days incubation.

Isolates		Characteristics of colony				DI
Code	Origin	Color	Elevation	Edge	Shapes	
P 1.1	ENT	White	Arising	Smooth	Oval, with the edge arising	1.20
P 1.2	ENT	Yellow	Arising	Smooth	Wrinkled	1.50
P 1.3	ENT	White	Like crater	Smooth	Oval, with the edge arising	1.10
P 1.4	ENT	White	Arising	Smooth	Round	1.70
P 1.5	ENT	White	Like crater	Smooth	Oval, with the edge arising	1.08
P 2.1	ENT	White, surrounded by pink color	Arising	Wavy	Complex (like flower)	1.10
P 2.2	ENT	White, yellow as the center	Like crater	Smooth	Oval, with the edge arising	1.09
P 2.3	ENT	White	Like crater	Smooth	Oval, with edges shellfish	1.11
P 2.4	ENT	White	Like crater	Wavy	Oval, with edges shellfish	1.22
P 3.1	ENT	Brownish yellow	Arising	Irregular	Oval, with edges shellfish	1.27
P 3.2	ENT	Brownish yellow	Arising	Wavy	Oval, with edges spread	1.50
P 3.3	ENT	Brownish yellow	Arising	Irregular	Oval, with edges spread	1.56
P 3.4	ENT	Brownish yellow	Arising	Irregular	Irregular and spread	1.25
P 3.5	ENT	Brown	Arising	Irregular	Oval, with edges shellfish	1.78
P 3.6	ENT	Yellow	Like crater	Wavy	Oval, with edges shellfish	1.50
P 4.1	ENT	White, yellow as the center	Like droplet	Smooth	Oval	1.08
P 4.2	ENT	White	Like crater	Smooth	Oval, with the edge arising	1.08
P 4.3	ENT	White, yellow as the center	Like crater	Smooth	Oval, with the edge arising	1.09
P 4.4	ENT	White	Like droplet	Smooth	Oval	1.08
P 5.1	WNT	White	Like crater	Wavy	Oval, with the edge arising	1.10
P 5.2	WNT	White	Arising	Wavy	Oval, with the edge arising	1.42
P 5.3	WNT	White	Arising	Smooth	Oval	1.57
P 6.1	WNT	White, yellow as the center	Like droplet	Smooth	Oval	1.25
P 6.2	WNT	White	Like crater	Smooth	Oval, with edges shellfish	1.22
P 7.1	CK	Yellow	Arising	Wavy	Oval, with edges spread	1.11
P 8.1	CK	White	Arising	Smooth	Oval, with edges shellfish	1.67
P 8.2	CK	Clear white	Flat	Smooth	Oval	1.10
P 9.1	CT	Brownish yellow	Arising	Smooth	Oval	1.62
P 10.1	CT	Brownish yellow	Flat	Smooth	Oval	1.80

Note: DI = Dissolution Index, WNT = West Nusa Tenggara, ENT = East Nusa Tenggara, CK = Cikabayan (IPB Experiment Station), and CT = Citeureup).

While the isolates that produced the lowest phosphatase enzyme were P 7.1 isolates, in which 0.17 mg kg^{-1}. Isolates P 3.3 and P 7.1, respectively, were isolated from samples of river sand Pinti, East Nusa Tenggara (ENT) and rhizosphere soil samples of soybean plants in IPB Cikabayan Experiment Station (Bogor, West Java).

Based on data in Table 2, it was also known that PSB isolates that produced the highest IAA in phosphate source $Ca_3(PO4)_2$ was isolates P 3.4, that was equal to 74.45 mg kg^{-1}. While the isolates that produced the lowest IAA was isolates P 1.1 isolates that containing 0.04 mg kg^{-1}. Isolates P 3.4 and P 1.1 were isolated from samples of river sand Pinti, East Nusa Tenggara (ENT) and rhizosphere soil samples of mahogany, East Nusa Tenggara (ENT), respectively. At the source of phosphatase $Ca_5(PO_4)_3OH$, isolates that produced the highest

IAA was isolates P 3.5, with the IAA content of 97.52 mg kg^{-1}. While the isolates that produced the lowest IAA was isolates P 2.3 in which 1.00 mg kg^{-1}. Isolates P 3.5 and P 2.3 were isolated from samples of river sand Pinti, East Nusa Tenggara (ENT) and rhizosphere soil samples of paddy fields, East Nusa Tenggara (ENT), respectively. Based on the results of Gram staining, it is known that all isolates of the BPF are a Gram-negative bacteria.

From the data in Table 2, three isolates were selected based on the content of the enzyme phosphatase and plant growth regulators IAA production which were highest in the origin area of each soil sample (Bogor, NTB, and NTT), the results are shown in Table 3. Selected isolates of PSB were then carried out further testing, the measurement of population growth, changes of pH in the Pikovskaya liquid medium, and phosphatase enzyme content during the 7 days of incubation.

The different productions of phosphatase enzyme and IAA of PSB were caused by characteristics of PSB that were isolated on different place. This is suitable with research by Karagoz *et al*. (2012) which showed that the different geographical locations, soil pH, and vegetation types in the investigated sites resulted in the different bacterial population and bacterial type. According to the research by Mander *et al*. (2012) the frequency of P-solubilisation in the bacterial population was significantly greater ($P < 0.001$) in soils of low-P status, demonstrating a selection pressure for this trait based on soil P availability. So, the abundance and composition of P-solubilising bacteria under strong selection pressure are affected

Table2. The ability of PSB in produces phosphatase enzyme and IAA during the 3 days incubation and its gram staining.

Isolates	Phosphatase enzyme (mg kg^{-1})		IAA (mg kg^{-1})		Gram staining
	$Ca_3(PO4)_2$	$Ca_5(PO_4)_3OH$	$Ca_3(PO4)_2$	$Ca_5(PO_4)_3OH$	
P 1.1	42.78	17.13	0.04	8.83	Gram negative
P 1.2	*	17.57	*	5.78	Gram negative
P 1.3	28.65	43.44	*	16.22	Gram negative
P 1.4	30.61	8.44	2.24	3.61	Gram negative
P 1.5	30.39	14.96	*	11.87	Gram negative
P 2.1	5.39	21.26	*	8.39	Gram negative
P 2.2	24.74	17.35	*	14.04	Gram negative
P 2.3	24.09	18.44	*	1.00	Gram negative
P 2.4	42.57	24.96	*	10.57	Gram negative
P 3.1	63.00	9.30	7.59	45.35	Gram negative
P 3.2	34.74	242.13	5.27	4.04	Gram negative
P 3.3	98.22	2.13	38.99	1.87	Gram negative
P 3.4	92.35	3.44	74.45	18.83	Gram negative
P 3.5	76.70	144.52	3.06	97.52	Gram negative
P 3.6	50.83	6.04	37.59	13.61	Gram negative
P 4.1	36.70	31.48	*	*	Gram negative
P 4.2	9.52	21.48	*	1.87	Gram negative
P 4.3	39.30	18.44	*	12.30	Gram negative
P 4.4	41.48	16.26	*	2.30	Gram negative
P 5.1	21.70	47.13	*	2.74	Gram negative
P 5.2	41.70	17.78	*	*	Gram negative
P 5.3	28.44	20.61	*	17.09	Gram negative
P 6.1	31.26	10.61	*	3.61	Gram negative
P 6.2	66.70	43.44	*	15.35	Gram negative
P 7.1	0.17	14.09	*	1.44	Gram negative
P 8.1	21.70	14.52	*	8.39	Gram negative
P 8.2	29.52	21.48	*	2.74	Gram negative
P 9.1	31.26	70.39	7.96	1.44	Gram negative
P 10.1	30.17	100.17	20.13	94.04	Gram negative

*= Not detected.

Table 3. Selected isolates of PSB with highest phosphatase enzymes and IAA production in each region of origin soil samples.

Isolates		Phosphatase enzyme (mg kg^{-1})		IAA (mg kg^{-1})	
Code	Origin	$Ca_3(PO_4)_2$	$Ca_5(PO_4)_3OH$	$Ca_3(PO_4)_2$	$Ca_5(PO_4)_3OH$
P 3.5	ENT	76.70	144.52	3.06	97.52
P 6.2	WNT	66.70	43.44	*	15.35
P 10.1	Citeureup, Bogor	30.17	100.17	20.13	94.04

*= Not detected.

by farm management strategies. Better understanding of their ecology provides the opportunity to increase the availability of soil P for plant-uptake.

Population Growth, pH, and Phosphatase Enzyme Content on Selected PSB

The three isolates of selected PSB that were P 3.5 which was derived from a soil sample of East Nusa Tenggara (ENT), P 6.2 which was derived from a soil sample of West Nusa Tenggara (WNT), and P 10.1 which was derived from soil samples Citeureup (Bogor, West Java) were done further testing, namely (i) population growth, (ii) changes of pH in the Pikovskaya liquid medium, and (iii) the content of the enzyme phosphatase. The all tests were conducted for 7 days of incubation. Population growth of each selected PSB isolate is presented in Figure 1.

Figure 1 shows that all three isolates had a phase lag or beginning phase on day-0 to day-1. The stationary phase was occured on day-3 to day-4. While the death phase was occurred from the day-5 to day-6.

Changes of acidity he media is one indicator of cell metabolic activity. Research by Perez *et al.* (2007) sowed that 10 of 130 of the PSB isolates were able to mediate almost complete solubilization of Ca3(PO4)2 in liquid cultures Acidification of culture supernatants were likely to be the main mechanism for P solubilization. This was suitable with research by Yu *et al.* (2012) which showed the strong correlation was found between pH and soluble P concentration, as well as the total organic acid production and P solubilization by PSB. This was suitable with research by El-Tarabily *et al.* (2008) which showed that a significant ($P < 0.05$) reduction in pH and a concurrent significant ($P < 0.05$) increase of the released P concentration were detected in the broth of the phosphate solubilizing isolates in PRP-amended broth compared to the non-phosphate-solubilizing isolate. Research by Muleta *et al.* (2013) showed that solubilisation of hydroxyapatite (HAP)/tricalcium phosphate (TCP)

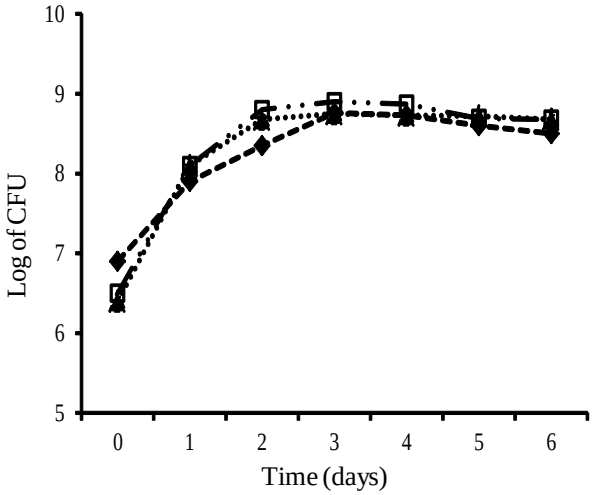

Figure 1. The population growth of selected PSB in Pikovskaya medium with a $Ca_3(PO_4)_2$ as a source of P. -◆- = P 3.5, -□- = P 6.2, and ···▲··· = P 10.1.

by all isolates were coincided with a decrease in medium pH and the production of organic acids by kind of phosphobacteria that could be considered as the major mechanism involved in the solubilisation of insoluble HAP/TCP. Value of pH on Pikovskaya liquid medium for all three isolates were likely to decrease (Figure 2).

The decrease in medium pH was caused by secreting organic acids that was acquitted by a number of PSB in the activity. Phosphate solubilizing bacteria will deliver a number of organic acids such as citric acid, glutamic, succinic, lactic, oxalic, glicooxalate, malic, fumaric, tartaric acid, α-ketobutirat. Increasing organic acids are usually followed by a sharp decrease in pH, thus resulting in the dissolution of Ca-P (Subba-Rao 1982). Rachmiati (1995) argued that each type of PSB has the ability to produce genetically different in the number types of organic acids that play a role in determining the level of dilution P.

Phosphatase enzyme content tended to increase until day 4, then decreased on day-5 but increased again on day-6 (for isolates P 3.5 and P 10.1). As for the isolates P 6.2 increasing of the enzyme phosphatase content did not so sharp as compared

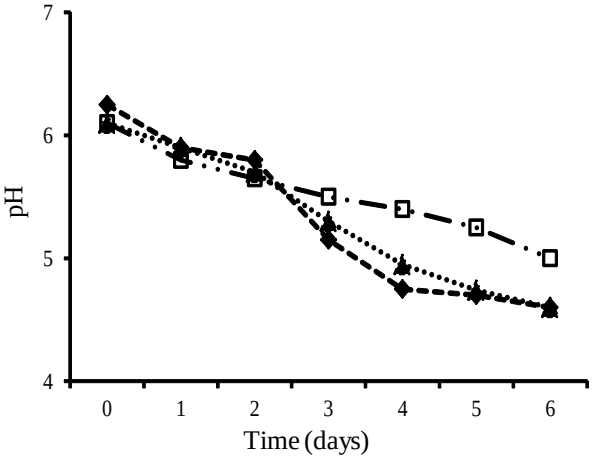

Figure 2. The changes in pH of selected PSB in Pikovskaya medium with a $Ca_3(PO_4)_2$ as a source of P. -◆- = P 3.5, -□- = P 6.2, and -▲- = P 10.1.

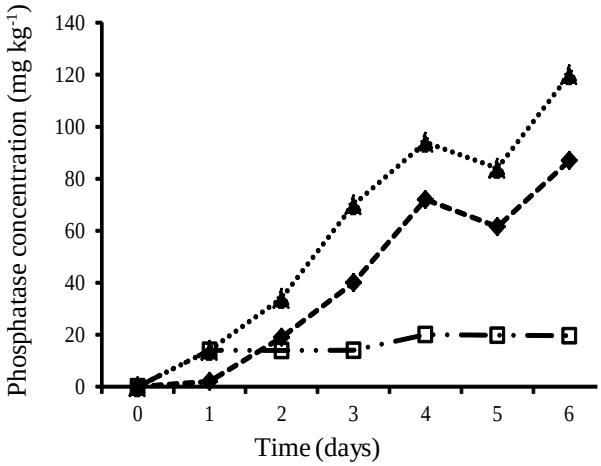

Figure 2. Phosphatase concentration on selected PSB in Pikovskaya medium with a $Ca_3(PO_4)_2$ as a source of P. -◆- = P 3.5, -□- = P 6.2, and -▲- = P 10.1.

to isolates of P 3.5 and P 10.1. Isolates of P 1.10 had the highest content of a phosphatase enzyme followed by isolates of P 3.5 and P 6.2.

Molecular Identification of Phosphate Solubilizing Bacteria

Deoxiribonucleat Acid has been isolated and tested to detect the presence of DNA using agarose gel electrophoresis. Having obtained the DNA, the PCR was then performed. The result of PCR amplification PSB isolates using 16S rRNA primers that produced a PCR amplicon or product was about 1,300 bp (Figure 4). Amplicon was subsequently sequenced to determine the nucleotide sequence of the 16S rRNA gene of each isolate.

Based on the results of 16S rRNA gene, sequence analysis of the program WU-BLASTN

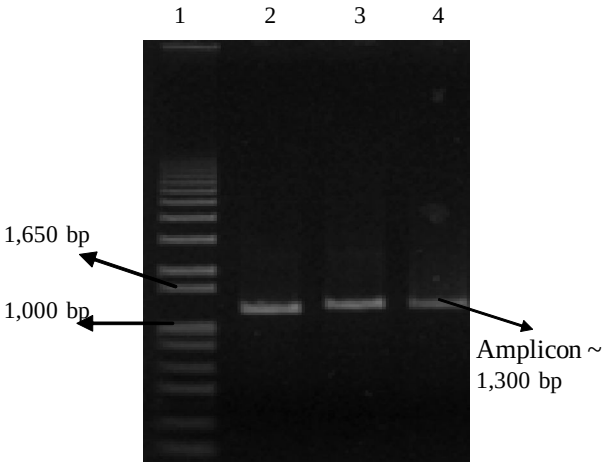

Figure 4. The result of amplification 16s r RNA gene from PSB. 1 = 1 kb DNA ladder marker, 2 = isolate P 3.5, 3 = isolate P 6.2 and 4 = = isolate P 10.1.

2.0 homology was done to know species from three isolates of PSB. Isolates of P 3.5 (ENT) and isolates of P 10.1 (Citeureup) had a similarity of 98% with *Gluconacetobacter* sp. strain RH1-MS-CO, whereas isolates of P 6.2 (WNT) had a similarity of 97% with *Enterobacter* sp. pp9c strain. Based on research by Ribeiro *et al.* (2012), analysis of fatty acid profiles on PGPR by partial sequencing of the 16S rRNA gene produced approximately 550–650 base pairs and the evaluation of the similarity of the bacteria also showed the same three families: *Bacillaceae, Enterobacteriaceae and Pseudomonadaceae.* The results of research by Azzis *et al.* (2012) showed that two hundred and fifty PSB were isolated in 2007 and they were classified according to their phosphate solubilization activity *in vitro*. Twelve of these isolates showing the greatest solubilization activity were selected for 16S rDNA sequencing. Ten isolates were presumably belong to the genus *Pseudomonas* and two isolates showed high similarity with members of the genera *Burkholderia* and *Acinetobacter.*

CONCLUSIONS

A number of 29 phosphate solubilizing bacteria isolates were obtained from soil at Experiment Station of Cikabayan IPB (Bogor, West Java), Citeureup (Bogor, West Java), West Nusa Tenggara (WNT), and East Nusa Tenggara (ENT). Based on the ability to produce enzyme phosphatase and to obtain the highest IAA three isolates were selected, namely isolates of P 3.5 (ENT), P 6.2 (WNT), and P 10.1 (Citeureup) and all isolates of bacteria were a Gram-negative bacteria. Isolates of P 10.1 had the highest ability to dissolve P with the largest dissolution index (IP), in which 1.80, the

highest phosphatase enzyme content (120.40 mg kg^{-1}), and the highest pH decrease. The results of 16S rRNA gene sequence analysis showed that isolates of P 3.5 and P 10.1 had 98% similarities to *Gluconacetobacter* sp. strain RH1-MS-CO on the GenBank data, while the isolates P6.2 had a 97% similarity to *Enterobacter* sp. pp9c strain on the GenBank data.

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