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Production of Acid Phosphatase in *Bacillus* sp. Isolated from Forest Soil of Gunung Salak National Park

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ABSTRAK

Produktivitas Fosfatase Asam pada *Bacillus* sp. yang Diisolasi dari Tanah Hutan Taman Nasional Gunung Salak. Pada pengamatan ini dilakukan karakterisrik bakteri pelarut fosfat yang diisolasi dari tanah hutan Taman Nasional Gunung Salak. Sebanyak 21 koloni hasil isolasi diuji terhadap produktivitas enzim fosfatase berdasar pelarutan media mengandung fosfat. Isolat yang terkuat melarutkan fosfat diidentifikasi sebagai *Bacillus* sp. Pada pengamatan lanjutan terhadap strain teruji dilakukan penumbuhan pada media cair selama 90 jam inkubasi, dan hasilnya ternyata mampu melarutkan fosfat inorganik (Pi) dari sumber trikalsium fosfat (Ca-Pi) dan alumunium fosfat (Al-Pi) masing-masing pada kisaran 1,2 sampai 152 dan 0.8 sampai 25 $mg.L^{-1}$; dan menunjukkan aktifitas enzim fosfomonoesterase antara 0.2 sampai 1.01 unit pada media yang mengandung larutan *para-nitrophenylphosphate* sebagai media fosfat organik (Po) artifisial. Konsumsi glukosa pada media yang diukur selama pertumbuhan sejalan pula dengan produk ortofosfat sebagai akibat adanya aktifitas enzim fosfatase. Peningkatan fosfatase juga sejalan dengan bertambahnya biomassa sel bakteri dan penambahan produk asam glukonat. Penurunan pH dari 7 menjadi 5 diakibatkan peningkatan produk asam glukonat di dalam media tumbuh. Bakteri pelarut fosfat yang berasal dari tanah hutan Taman Nasional Gunung Salak dapat memproduksi fosfatase asam untuk memineralisasi sumber-sumber fosfat menjadi sumber nutrisi yang siap digunakan oleh akar tumbuhan, dan itu merupakan prediksi kuat untuk menjadikan isolat bakteri pelarut fosfat sebagai sumber bahan pupuk hayati.

Kata kunci: *Bacillus* sp., tanah hutan, fosfatase asam, Ca-Pi, Al-Pi.

INTRODUCTION

Ecology of microbial communities can be attributed in part to understand that these organisms have directly effects on ecosystem processes (Beare *et al.* 1995; Horner-Devine *et al.* 2004; Fierer & Jackson 2006). Phosphate solubilizing bacteria (PSB) commonly found as adaptive bacteria (Glenn & Mandelstam 1971) in most soils (Chonkar & Taraedar 1984; Venkateswarlu *et al.* 1984). The population levels of phosphobacteria were higher in the rhizosphere soil, and able to produce phytohormones and phosphatases enzyme under in vitro conditions (Ponmurugan & Gopi 2006). PSB was able to convert the insoluble phosphates into soluble forms by acidification, chelating and exchange reactions, and production of gluconic acid (Chen *et al*.

2006). The soluble forms also may contribute to their stimulatory effect on plant growth (Hameeda *et al.* 2006).

Correlation between PSB growth and capacity of phosphatases enzyme activity, as due to availability of phosphorous content in the medium identified by Barik & Purushothaman (1998). In the other hand, there is increasing evidence that phosphobacteria improve plant caused to biosynthesis of plant growth substances rather than their action in releasing available phosphorous. Subsequently, PSB is requisite for land reclamation and restoration to improve despoiled land since the genus has useful producing plant growth promoter, phosphorus availability to plant, and as the competitor of plant bacterial pathogen.

In the preliminary work, the fastest growing and the widest clearing zone formation in selective agar media for the isolate of PSB has deprived from forest soil collected from Gunung Salak National Park. In the existing investigation, culture approach has been studied whether the fine isolate and then delineated as gram-positive bacterium, named *Bacillus* sp. Some researcher find out that the genus relatively has large number of protein phosphatases (Cohen 1989; Villafranca *et al.* 1996), it can be serine/threonine phosphatases which have showed wide specificities, and also tyrosine phosphatases.

The present work was undertaken to compare the phosphate-repressible enzyme formed by the *Bacillus* sp. Activity of phosphatases enzymes evaluated through the bacterial growth within media containing tri-calcium phosphate and aluminum phosphate as inorganic phosphate, and in the bacterial growth containing para-nitrophenyl phosphate (*p*Npp) as organic (artificial) phosphate. The results suggest that the phosphate mineralization capability of the PSB would appropriate to produce plant nutritive value in subsequent work on biofertilizer function. The presence of PSB in Gunung Salak National Park is not only important for ecosystem health but also important genetic resources.

MATERIALS AND METHODS

Surface soil samples (up to 20 cm depth) collected from fields as a bulk samples of various places at forest floor in Gunung Salak National Park; the altitude is 900 m above sea level; situated around S $06^0$46’24.3" - $06^0$46’49.8" and E $106^0$42’09.9" - $106^0$42’25.9"; in June 2009. Composite soil sample of each soil case mixed thoroughly, with then air dried and passed throughout 100 mesh sieves for studies.

In the preliminary work, based on the broadest halozone screen ability growing in selected media, the 21 isolates recognized as PSB (Tabel 1). Selected isolate (GS1) then identified through analysis of gene 16S RNA using the method of Pitcher *et al.* (1989), and identified as *Bacillus* sp.

In the further quantification, the strain cultured on liquid media contains of phosphorous substances, and incubated on a rotary shaker (180 rpm) at 30ºC. For liquid media, mineral phosphate (5 $g \cdot L^{-1}$ tri-calcium phosphate or aluminum phosphate) were sterilized

separately and then mixed with the autoclaved medium (10 g·L^{-1} glucose; 0.27 g·L^{-1} $NH_4 \cdot NO_3$; 0.2 g·L^{-1} KCl; 0.1 g·L^{-1} $MgSO_4 \cdot 7H_2O$; 1 mg·L^{-1} $MnSO_4 \cdot 6H_2O$; 1 mg·L^{-1} $FeSO_4 \cdot 7H_2O$; and 0.1 g·L^{-1} Yeast extract).

Glucose oxidation to become gluconic acid is a major mechanism for mineral phosphate solubilization. When gluconic acid content and phosphomonoesterase activity in the culture medium measured, the strain was prepared for preculturing in LB medium (10 g·L^{-1} polypeptone, 5 g·L^{-1} NaCl, 5 g·L^{-1} Yeast extract, 1 g·L^{-1} glucose). The precultured was washed twice with 10 mM potassium phosphate buffer (pH 7.5) and resuspended in the same buffer and at the same concentration as the original pre-culture. The suspension (inoculum mass 5%, v/v) was then transferred to 100 ml glucose minimal medium containing 0.4% glucose and 21 mM potassium phosphate buffer (pH 6.8); and incubated on a rotary shaker (180 rpm) at 30ºC. For determination of organic acid produced, the strain was cultured in GMS medium containing 10 g·L^{-1} glucose; 2 g·L^{-1} $(NH_4)_2SO_4$; 0.3 g·L^{-1} $MgCl_2 \cdot 6H_2O$; 1 mg·L^{-1} $MnCl_2 \cdot 4H_2O$; 6

Tabel 1. PSB was common bacteria isolated from Gunung Salak National Park

No	Strain	[a]Growth rate	[b]Ca-P dissolution capacity
1	GS1	+++	3.1
2	GS2	++	2.8
3	GS3	++	2.8
4	GS4	+	1.9
5	GS5	+	1.8
6	GS6	+	1.8
7	GS7	+	1.8
8	GS8	+	1.8
9	GS9	+	1.7
10	GS10	+	1.7
11	GS11	+	1,7
12	GS12	+	1,7
13	GS13	+	1,6
14	GS14	+	1.7
15	GS15	+	1.7
16	GS16	+	1,7
17	GS17	+	1,7
18	GS18	+	1,6
19	GS19	+	1.7
20	GS20	+	1.7
21	GS21	+	1,7

Remarks: +++ fast growing; ++ medium growing; + slow growing, [a] Colonies appear after 24 h (+++); after 48 h (++); and after 72 h (+). [b] Ratio of clear zone areas per colony areas

mg·L^{-1} $FeSO_4 \cdot 7H_2O$; 6 mg·L^{-1} $NaMoO_4$; thiamine (20 ì·L^{-1}); tri-calcium phosphate (20 mg·mL^{-1}); and incubated on a rotary shaker (180 rpm) at 37ºC.

Cells were collected by centrifugation at different growth periods in order to verifications the change in pH and P-concentration in the medium. Samples were centrifuged 6000 rpm for 10 minutes to receive clear solutions for analysis. The P-concentration and pH were determined throughout supernatants in each investigation. The P-concentration estimated with ascorbic acids methods. The 50 ~ 200 micro-liter of the culture filtrate was mixed with 900 micro-liter of phosphorus mixed reagents, keep at room temperature for 10 minutes. The P-concentration was measured in spectrophotometer at 880 nm. In order to observe the effect of cultural conditions for mineral phosphate solubilizing, bacterial strains were cultured at various insoluble phosphates (tri-calcium phosphate, and aluminum phosphate) at defined concentrations of glucose conditions.

Five ml of a precultured solution was inoculated in 50 ml of medium in a 250-ml Erlenmeyer flask. After inoculation, the flasks were placed on a shaker and the bacteria were grown at 37^0C for 24 h. The supernatant of each culture was obtained by centrifugation at 10,000 rpm for 10 min. For the experiment to determine gluconic acid, a 0.5 ml aliquot of the culture filtrate passed through 0.2 ìm Whatman membrane filter. The organic acids in filtrates were identified by high-performance liquid chromatography with a Thermo hypersil C18 column (250 x 4.6mm). Organic acids were monitored using a UV detector at 220 nm. The mobile phase consisted of 50 mM sodium phosphate and 5 mM tetra-butyl-ammonium hydrogen sulfate, pH 6.5 (95%), plus acetonitrile (5%) and a flow rate of 0.25 ml·min^{-1}.

The protein phosphatases activity was tested by the ability to hydrolyze *p*-nitrophenyl phosphate (pNpp) in a buffer containing 50 mM Tris HCl (pH 7.2). Phosphomonoesterase activity (PME) was measured after incubation at 37ºC (Margesin1996). Phosphatases activity was determined by measurement of *p*-nitrophenol in a spectrophotometer at a wavelength of 400 nm. PME activity was expressed in unit and defined as micromoles nitrophenol produced by 1 ml enzyme per hour.

Three replicate of flasks and tubes were used for all in each treatment of examination; and also from which the other samples were collected at 4 to 96 hours in various sampling interval of incubation; those were analyzed for the parameters studies. Analysis of correlation and some other statistical requirement among mean values calculated at various variables amongst the confidence level of the degree of freedom (Parker 1979).

RESULTS

The isolated bacterium had a marked of insoluble phosphate solubilizing activities, because the culture grows to visualize clear zone upward in the region of the colony forming after 3 days

incubation at 30ºC. In estimating the efficacy of phosphate source utilization by the PSB, result in phosphatases assay of *Bacillus* sp. in liquid cultures containing insoluble phosphate analytically measured. Result of the studies have correlated in the parameters measurement of Ca- and Al-phosphate metabolism as inorganic phosphate incorporation to glucose reduction in the substrate, but it does not proper to organic phosphate of para-nitrophenylphosphate. The highest production of phosphorus substance during incubation was measured, and it found in the medium containing tri-calcium phosphate. Gluconic acid production was increase followed by pH reduction in the culture medium (Table 2). This indicates the evenness of phosphatic source utilization, a prominent phenotypic characteristic of phosphate solubilizing bacterial isolates in relation to carbon augmentation.

Most bacterial (PSB) population was stable after 24 hours incubation. PSB live activity in culture was closely followed by gluconic acid production as caused by glucose incorporation. The phenomenon gives sequence that the option culture incubation for *Bacillus* sp. should be after 36 hours and reaching the limit action in 60 hours incubation (Figure 1). That information becomes useful to find out the maximum incubation period for that bacterial in the propagation culture for biotechnological purpose.

Kinetic potential of unit phosphatase activities belonging to *Bacillus* sp. was measured in tri-calcium phosphate and alumunium phosphate solution culture substance; and subsequently those measurement acquiesced phosphate quantity of culture in the yield of 150 and 25 $mg \cdot L^{-1}$ respectively, at 88 hours incubation (Figure 2). The result assumed the PSB effectively capable to increase soluble phosphate in their habitat (in soil of forest floor in Gunung Salak National Park), because of phytase commonly

Table 2. Probability levels for statistical significance value in observation

Parameters studied of in-*vitro* culture of *Bacillus* sp.	**Correlation**
1. Relationship of Ca-phosphate solubilization vs. glucose reduction in the substrate:	y = -24.04x + 3832.7 (r = - 0.91 S)*
2. Relationship of Al-phosphate solubilization vs. glucose reduction in the substrate:	y = -199.07x + 4890.1 (r = - 0.95 S)*
3. Relationship of Ca-phosphate to Al-phosphate solubilization in the substrate:	y = 23x + 0.38 (r = 0.37 NS)*
4. Relationship of gluconic acid production to pH level for the period of incubation:	y = -0.78x + 7.27 (r = - 0.97 S)**

Remarks: * (S) Significant and (NS) non-significant at $p_{0.001} = 0.597$; df = 25, with 26 samples ** (S) Significant at $p_{0.001} = 0.872$; df = 8, with 9 samples

have specify requirement to Ca^{3+} and Al^{2+} for its enzyme activity.

Phytate is the major component of organics forms of P in soil (Richardson 1994). Phytase activity can be important for stimulating growth under limited P in soil, and supports to improve or transfer the P-solubilizing trait to plant-growth-promoting bacteria (Rodrý´guez *et al.* 2006). Most phytases (myo-inositol hexakisphosphate phosphohydrolases) belong to high molecular weight acid phosphatases, which has capability to hydrolyze of phytic acid to be orthophosphate inorganic and phosphate esters from lower mio-inositol. Phytic acid is a phosphate ester that usually establishes in soil, and it could bond the important minerals and protein. Phosphomonoesterase (PME) examined as acid phosphatase under Tris-HCl (pH 7.2) buffer in the medium culture solution, but only low activity of the enzyme detected in the investigation.

DISCUSSION

Microbial communities in forest soil play important role in maintaining global ecosystem health. PSB is one of the important soil microbes which stimulate dissolution of less soluble-P into soluble form and that available for plant growth. The availability of P in soil is generally low, and in forest ecosystem most of phosphate in the form of organic bound phosphate. Those organically bound phosphates should be hydrolyzed by phophatase enzyme produced by soil microorganism. Soluble phosphate released ($H_2PO_4^-$, $HPO_4^=$ and $PO_4^=$), are then undergoes several biochemical

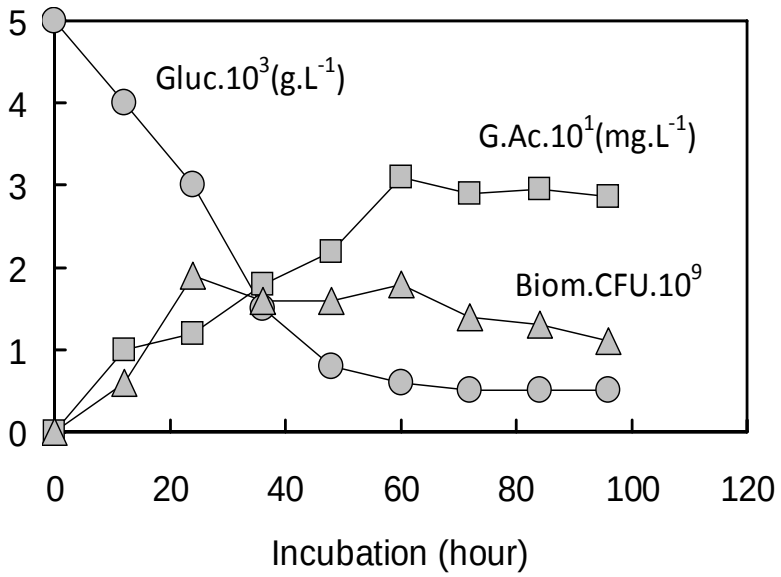

Figure 1. *Bacillus* sp. ( ) increased which indicate apart of glucose ( ) consumed; and it was converted into biomass followed by gluconic acid ( ) increasing in the media

transformation, or again bounded to other soil minerals (Cunningham & Kuiack 1992). The availability of phosphate in soil is greatly dependent on soil pH, carbon content, redox potentials, and soil physical structures. The Strain of GS1 identified as *Bacillus* sp., was able to solubilize both Ca-P and Al-P. Their ability to solubize those less soluble phosphate may indicate that the strain is important in stimulating phosphate dissolution. The physiological mechanism by which P dissolved is very complex.

Phosphobacteria (PSB) have been found to produce some organic acids such as monocarboxylic acid (acetic, formic), monocarboxylic hydroxy (lactic, glucenic, glycolic), monocarboxylic, ketoglucenic, decarboxylic (oxalic, succinic), dicarboxylic hydroxy (malic, maleic), and tricarboxylic hydroxy (citric) acids in order to solubilize inorganic phosphate compounds (Lal 2002). Phosphobacteria deprive from rhizosphere soils when was tested under in-vitro condition on their production capacity of growth regulators and phosphatase enzyme (Ponmurugan & Gopi 2006). Several soil bacteria posses the ability to solubilizing insoluble inorganic phosphate and make it available to plants. The effect is generally due to the production of organic acids by these organisms, and also produce amino acids, vitamins, and growth promoting substances like indole acetic acid (IAA) and gibberellic acid (GA_3), which help in better growth of plants (Richardson 2001;Gyaneshwar *et al*. 2002). The results of enzyme activities, including soil phosphatase activity, could be compared not only with soil physical and chemical properties, but also with other biological

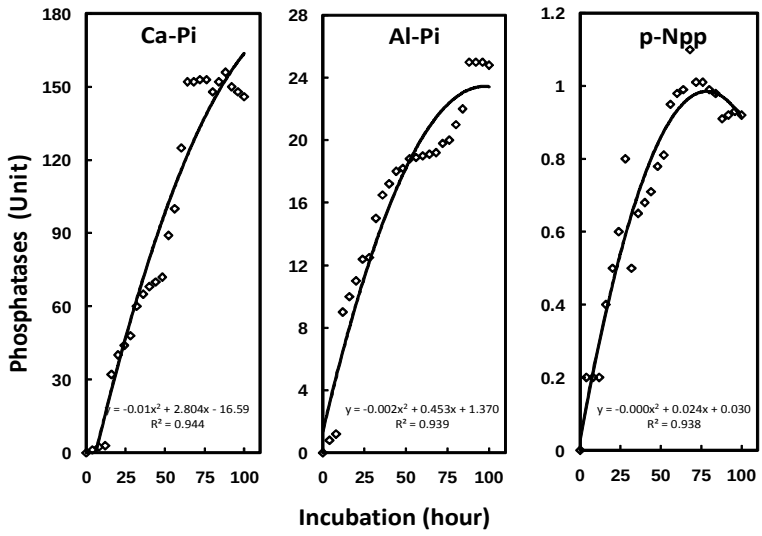

Figure 2. Trend of kinetic potential of phosphatase released with *Bacillus* sp. altering phosphate inorganic (Ca- and Al-phosphate; Figure 2A & 2B), and phosphate organic (*p*-nitrophenyl-phosphate; Figure 2C)

factors such as microbial biomass, the level of adenosine triphosphate, etc. (Chhonkar & Tarafdar 1984). Falih & Wainwright (1996) found that the activity of phosphatase enzymes increased when a carbon source was added to the soil. Evaluate to the result of experiment here, inform that glucose incorporation proved to cause increasing cell biomass of *Bacillus* sp. in culture, and gluconic acid increment (Figure 1); and in the different way, the medium acidity is plunge to 5 from pH 7 as before during 96 hours incubation.

Alkaline phosphatase of several bacterial species has been investigated by Landeweert *et al.* (2001), and the genus have differ one from another in certain respects. The genus seems in general to share the property of being repressed by phosphate inorganic in *Escherichia coli, Bacillus subtilis, Pseudomonas fluorescens* and *Staphylococcus aureus*. PSB is capable of solubilizing accumulated insoluble phosphate compound sources in soil by the production of organic acid, phenolic compounds, protons, and siderophores. According to Kelly *et al.* (1984) investigation's, maximum phosphates (inorganic pyrophosphate) formed at 12 hours incubation in *Bacillus* sp. when divalent metal Mn^{2+} occurred in the medium culture, and the inorganic pyrophosphates activity found as intracellular enzyme. In the other study on the extracellular phosphates activity, Nomoto *et al.* (1988) found the optimum alkaline phosphate excreted into broth culture fairly stable in pH 5 ~ 12. Extracellular enzymes, including phosphatases, are important for the degradation of organic substances in the soil for organic phosphate mineralization (Hysek & Sarapatka 1998). The functionalities of PSB communities differ on the basis of phosphate sources present in the culture medium in this investigation. Phosphatases activities expressed optimum in 75 to 100 hours incubation (Figure 2) may cause the $MnCl_2$ containing in media culture.

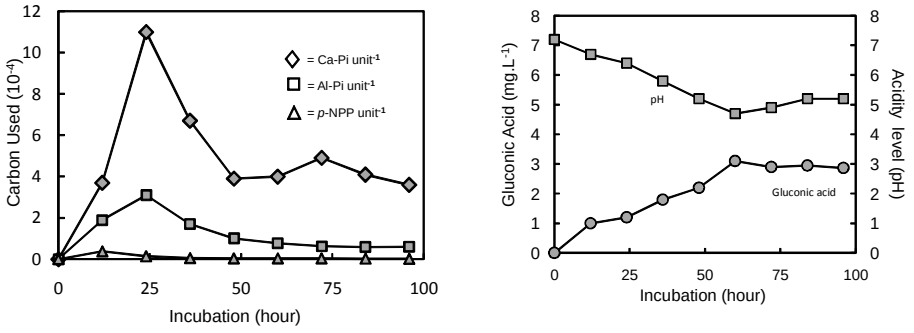

Figure 3. Incorporation of glucose along with phosphatases activities and carbon used by *Bacillus* sp. (left), followed by increasing of gluconic acid and pH 7 reduce to pH 5 (right)

Gluconic acid is organic acid compound arise from the oxidation of glucose. It is produce by the fermentation of glucose by bacteria, and in aqueous solution at neutral pH the gluconic acid forms the gluconate ion. Chen *et al.* (2006) investigate the isolates of phosphobacteria collected from agriculture soil. The isolates belong to genus of *Bacillus*, *Rhodococcus*, *Arthrobacter*, *Serratia*, *Chryseobacterium*, *Delftia*, *Gordonia* and *Phyllobacterium* were identified. In other finding, four strains namely *Arthrobacter ureafaciens*, *Phyllobacterium myrsinacearum*, *Rhodococcus erythropolis* and *Delftia* sp. reported for the first time as PSB after confirming their capacity to solubilized considerable amount of tricalcium phosphate in the medium by secreting organic acids. Ten isolates of *Bacillus megaterium* can do not producing any gluconic acid in the culture along 72 hours incubation. In the contrary, 96 hour's incubation of *Bacillus* sp. in this experiment was able to produce gluconic acid, and negatively correlated with glucose incorporation but positively correlated to cell biomass (Figure 3).

CONCLUSION

Isolate of *Bacillus* sp. demonstrate clearly in solubilizing tri-calsiumphosphate more than alumuniumphosphate; Increasing cell population of *Bacillus* sp. was following in gluconic acid yield as due to glucose metabolism as carbon source in culture; *Bacillus* sp. was certainly characterized on the basis of biochemical reaction, and as due to the cultural performance in the medium containing inorganic phosphate.

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