PHOSPHATASE ACTIVITY **OF** *Bacillus* **sp. ISOLATED FROM FOREST SOIL OF** GUNUNG **HALIMUN NATIONAL PARK**

[Aktifitas Fosfatase *Bacillus* sp. yang Diisolasi dari Tanah Hutan di Taman Nasional Gunung Halimun]

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ABSTRACT

Bacillus sp diisolasi dari tanah Taman Nasional Gunung Halimun. Dalam media tumbuh yang mengandung $Ca_3(PO4)_2$ bakteri membentuk zona bening disekitar koloni. Glukosa digunakan dengan cepat selama kultivasi. Tinggi konsentrasi fosfat terlarut yang dibebaskan selama fase pertumbuhan menunjukkan bahwa bakteri mampu memacu pelarutan $Ca_3(PO4)_2$. Selama fase pertumbuhan terjadi hidrolisa organik fosfat (phenyl phosphate) menghasilkan phenol dan fosfat hal ini menunjukkan, bahwa *Bacillus* sp mampu menggunakan organik fosfat. Selama kultivasi terjadi penurunan pH sejalan dengan pelarutan $Ca_3(PO4)_2$.

Kata kunci: *Bacillus,* aktifitas fosfat, Taman Nasional Gunung Halimun.

INTRODUCTION

Soil microflora play significant role in transformation of both either organic and inorganic phosphorous (Alexander, 1961). Soil microorganisms produce phosphatase enzymes enable the microorganism hydrolyze phosphorous and becomes available for plant growth. To determine the activity of phosphatase in soil some methods were proposed (Tabatabaii, 1982). Basically phosphate ester bound compound such as p-naftilphosphate, phenylphosphate, p-nitrophenylphosphate, bis p-nitrophenylphosphate, tris p-nitrophenylphosphate, and p-nitrophenyl phosphorylcholin. Of which P-naftilphosphate, phenylphosphate, p-nitrophenylphosphate is mostly used for determining phosphomonoesterase activity. The presence of those substances in solution stimulate the microbe produce enzyme and finally hydrolize the ester bound phosphate, and thereby the hydrolysis product is quantified. Phosphodiesterase hydrolyze nucleic acids and found in microorganism, plant and animal. The activity phosphotiestearse in soil was found in 1976, but not much attention is given further (Dick *et al.*, 2000). Phospholipid is a component of cell membrane of most organism containing cholin. Phospholiplase-C has capacity to hydrolyze lecithin to 1,2 digleceraldehyde, choline phosphorine, many bacteria has capacity to produce that enzyme. Many works have been devoted to verify the kinetic and characteristic of phosphomonoesterase (PME). Categorized as the hydrolase group enzyme owing to its capacity to introduce H_2O molecule substituting ester bonded phosphate. Mainly produced when soil limited in ionic phosphorous, of which excreted mainly by microbes and soil animal but rare by plant (Schiner *et al,* 1996). Bacterial cell P is mineralized quickly, phospholipid, and DNA are dephosphorelated in a short period. P in microbial RNA is released more slowly. Our present work aimed at studying the characteristic of PME produced by soil bacteria.

MATERIALS AND METHODS

Isolation and identification

Bacillus sp was isolated from soil of Gunung Halimun National Park using Pivoskaya medium contained : 5 g l"[1] Ca3(PO4)2 l"[1] , 10 g l"[1] glucose, 0.2 g l"[1] NaCl, 0.2 g l"[1] KC1, 0.0025 g 1" MnSO4.H2O, 0.1 g l"[1] MgSO4 7H2O, 0.0025 g 1" FeSO4 7H2O, 0.5 g l"[1] yeast extract. The isolated

bacterium was the n growth maintaining media NA for further physiological studies.

Enzyme assay

Several methods were available for PME activity assay (Tabatabai, 1978; Dick and Tabatai, 1986). Our present study used p-nitrophenyl phosphate as a model substrate and released p-nitrophenol was measured spectrophotometrically. The basic principle of PME assay, P-nitrophenyl phosphate is added into supernatant culture and incubated for 1 h at 37°C. P-nitrophenol released is extracted with NaOH, and then measured spectrophotometrically at 400 nm. Enzyme activity is expressed as umoll p-nitophenol released per 1 supernatant. PME assay also can be conducted using phenylphosphate as enzim substrate, phenol released are then quantified spectrophotometrically.

Glucose consumption

Amount of glucose utilized was determined by dinitrosalycycilic method (Miller, 1959), and culture growth was measured spectrophoto-metrically

RESULT

The activity PME ase and the population of PSB in soil of GHNP is shown Figure 1 indicating that PME ase play significant role in accelerating phosphate mineralization in soil, and those enzymes are mainly produced by PSO (Tabatabai, 1978).

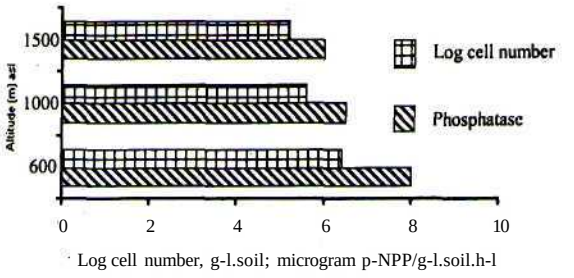

Figure 1. PME-ase activity and the population of phosphate solubilizing bacteria (Rahmansyah *et al.*, 2000).

PME activity is correlated with population of phosphate solubilizing organism implying tha: most of the phosphate mineralization in soil is conducted by microorganism .

pH

During culture growth pH was fluctuatec and they may affect the phosphate species in solution. At the beginning of incubation the pH o: culture decreased dramatically (Figure 2).

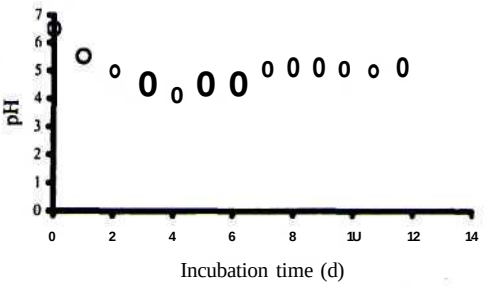

Figure 2. Profile of pH during culture incubation ir Pivoskaya Medium

Release of proton have remarkabK decreased pH value (equation 3). pH has potentia effect on the activity of enzymes by altering the functional active site of enzyme, altering solubilin of substrate, changing the adsorption rate, anc altering the substrate enzyme binding.

Growth of culture

Rapid growth of culture was observed ir Pivoskaya medium. The carbon sources utilizec mainly from glucose. After glucose was limited, cell growth was suppressed (Figure 3).

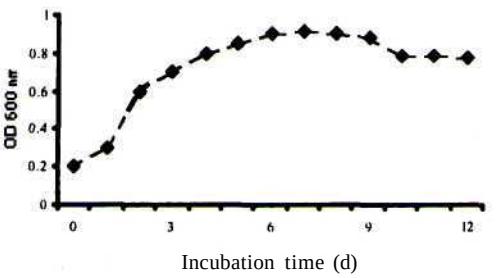

Figure 3. Growth of culture

Glucose

Many author used glucose as main carbon sources for phosphate solubilizing bacteria. Glucose was easily used by microorganism as indicated by a rapid decrease of glucose during cultivation. About 90 % of glucose was utilized (Figure 4). Glucose could be converted into reserve materials or for electron donor in fermentation processes for production of organic acids (Cosgrove, 1967).

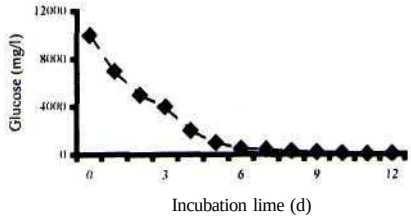

Figure 4. Glucose consumption during culture growth

Phosphate solubilization

Maximum P-released was after 2 days incubation was 2003 |ig/L (Figure 5). The relation between pH and P-dissolution is in consistent

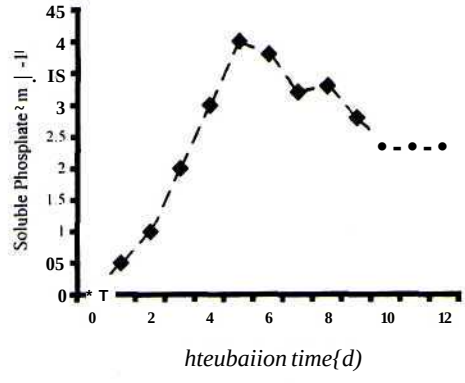

Figure 5. Phosphate released during culture growth

Activity PME ase

PME ase is a group of hydrolase enzyme which responsible for addition of H2O molecule into ester phosphate bound. The activity of PMEase was determined at pH 6.5, 38 C and incubated for 45 minutes. $CaCl_2$ was added to stop enzyme reaction and the activity is expressed as microgram />-NP/ml enzyme/ incubation time. And the specific activity is expressed as unit enzyme per mg protein. The Activity of PME-ase is shown in Figure 6. Maximum activity was observed when culture growth was maximum implying that enzyme activity was linked to cell growth.

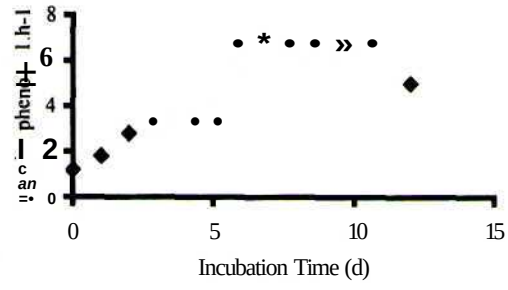

Figure 6. Activity of PME-ase

DISCUSSION

Many factor affecting availability of phosphorous to plant growth (Tabatabai, 1982). The presence of phosphate solubilizing organism (Table 1) which have capacity to solubilize P than it is necessary for its metabolism will enhance mineralization of phosphate in soil. Soil of GHNP contained a number of bacteria that are able to solubilize $Ca_3(PO_4)_2$ which was dominated by *Bacillus* sp is able to grow rapidly in Pivoskaya medium and forming clearing zone implying they are abfe to sofubiTi'ze inorganic phosphate (Alexander, 1961). Table 1 also indicates that PSO is quite diverse in soil consisted of yeast, bacteria and fungi.

Table 1. List of Phosphate Solubilizing organism (Rao, 1982).

Microorganism	Phosphate source
Bacteria	**Mineral**
- *Bacillus* sp., *B. fulvifaciens, B. megaterium, B. circulans, B. subtilis, B. mycoides, B. mesenlericus, B. fluorescence, B. circulans*	Tricalcium phosphate
- *Pseudomonas* sp., *P. Putida, P. liquifaciens, P. calcis, P. rathonia*	Calcium phosphate
- *Escherichia freundii, E. intermedia*	Iron phosphate
- *Xanthomonas* spp.	hydroxyapatite
- *Flavobacterium* spp.	Fluoroapatit
- *Brevibacterium* spp.	Rock phosphate
- *Serratia* spp.	
- *Alcaligenes* spp.	
- *Achromobacter* spp.	Organic
- *Aerobacter aerogenes*	Calcium phytate
- *Erwinia* spp.	
- *Nitrosomonas* spp.	Phytin
- *Thiobacillus thiooxidans*	Lecithin
	Phenyl phosphate
	Other organic phosphate
Fungi	
- *Aspergillus* sp.. *A. niger, A. jlavus, A. fumigatus, A. lerreus, A. awamori*	
- *Penicillum* sp., *P. lilacinum, P. digitatum*	
- *Fusarium* sp.. *F. oksisporum*	
- *Curvularia lunata, Humicola* sp., *Sderotium rolfsii*	
- *Pythium* sp., *Acrothecium* sp., *Phoma* sp.	
- *Mortierella* sp., *Paecilomyces* sp.	
- *Cladosporium* sp. *Rhizoctonia* sp.	
- *Cunninghamella* sp., *Rhodotulla* sp.	
- *Candida* sp.	
- *Schwanniomyces occidentalis*	
- *Oideodendron* sp.	
- *Pseudogymnoascus* sp.	
Actinomycetes	
- *Streptomycts sp.*	

One important aspect of soil pH is determining the oxidation state of phosphate in soil. The fluctuation of pH is chemically controlled by several mechanisms (Kpomblekou and Tabatabai, 1994). Lowering pH value in solution as a result of production of organic acids such as citric, glutamic acids, and oxalic acids (Kpomblekou and Tabatabai, 1994). Those organic acids bound with Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+} (Rao, 1982) result in released of ionic P. Released P will a part bound to Fe, Al and a part will be water soluble and thus available for plant. Released Ca, a part will bound with organic acids forming organic complex, and the remaining will bound with soil colloid, via cation exchange. When exchanged Ca increased result in increases of basic saturation, and thus increase pH values (Tabatabai, 1982). P-dissolution correlated with pH of culturing medium was observed by (Rao, 1982). Oxidation of ammonium in soil caused soil acidification (reaction 1)

$$22\ NH_4^+ + 37\ O_2 + 4\ CO_2 + HCO_3 \rightarrow C_5H_7N0_2 + 21NO_3 + 20H_2O + 42H^+ \quad (1)$$

Beside soil acidity, several mechanisms are reported to affect phosphorous dissolution. Cosgrove, 1967 proposed that proton (H^+) released from cytoplasm to outer membrane as an exchange

of NH_4^+ absorption, or releasing H^+ by ATP-ase utilizing energy derived from hydrolysis of ATP. Other mechanism could be direct solubilization of P took place in cell surface and produced soluble P in the form of H_2PO_4" and HPO_4^{2}" (Joner *et al*, 2000). Carbon dioxide produced during aerobic respiration may also affect P-dissolution follow reaction 2 and 3

$CO_2 + H_2O$ — -»HCO_3- + H^+ (2)

$Ca_3(PO_4)_2 + HCO_3$- --> 3 $Ca(HCO_3)_2$ + 2 PO_4^3- (3)

Those reactions took place in plant rhizosphere and released P is in excess than its necessary for bacterial growth (Rao, 1982).

Most of soil composed of more of organic-P than that of inorganic-P. Mineralization of organic-P executed by a complex of phosphatase enzyme that has capacity to hydrolyze ester phosphate and found both and intra-and extracelluler. Most of this enzyme is produced when the soil contain less of soluble phosphorous. Phosphatase is produced by microorganism and plant, but dominantly by microorganism. Tabataibai (1982) proposed 5 group of enzymes belonged to phosphatase: Phosphomonoesterase include Phytase, glycerin phosphatase, nucleotidase and sugar phosphatase. Phosphodiesterase such as nuclease and phospholipase. Phosphotriesterase. Poliphosphatase include ATPase and pirofostase inorganic. Phosphoamidase which breakdown phosphorous and nitrogen bound.

Phytic acids as the major component of soil organic-P hydrolyzed enzymaticaly by phosphomonoesterase especially phytase (Michael et al., 1994). Based on its optimum pH activity and substrate specificity phosphomonoesterase enzyme is grouped into acid and alkaline PME. Acid PME found in rhizosphere and plant tissue, in contrast alkaline PME only produced by microbes and soil animal. Fungi and bacteria produced both acid and alkaline PME (Joner et al., 2000). The activity of intracellular PME is higher in alkaline environment than that of neutral and acid. In contrast higher activity of extra-cellular PME is observed in acidic environment. Thereby activity of acid PME is high in acid soil and alkaline PME is high in alkaline environment.

The finding of phytin derivatives as well as as phytin itself in the soil organic fraction suggest that a breakdown of the inositol hexaphosphate take place. The enzyme phytase liberates phosphate from phytic acid or its calcium magnesium salt, phytin, with the accumulation of inositol.

Phytase activity is widespread and is enhanced by carbonaceus materials that increase the size of the microbial population. Species of *Aspergillus, Penicillium, Rhizopus, Cunninghamella, Arthrobacter, and Bacillus* can synthesize the enzyme (Rahmasyah et al., 2000). Yet, despite the great phytase potential, phytin is not readily metabolized in soil (Kpomblekou and Tabatabai, 1994). The hydrolyses apparently, is not limited by phytase producing capacity of microorganism, which is appreciable, but by the small amount of phytic acid in the soil solution. The fact that phytate-phosphorous is relatively unavailable to crop growing in acid soils where the substrate is bound into iron and aluminum complexes.

If the major reserves of P in humus were phytin and nucleic acids, it would seem that the later is the more active fraction in mobilization and immobilization reaction because phytin is relatively resistance to decay while nucleic acids are highly susceptible to microbial attack.

Attempt has been made to exploit microbiological phosphate release. When microorganism known as phosphobacteria is inoculated into soil or on the seeds of several crop plants, there allegedly is remarkable increase in yield and in P-content of the crop harvested.

Oxidation-Reduction Reaction

P like N, may exist in a number of oxidation states ranging from the -3 of Phosphine, PH3, to the oxidized state, +5, of orthophosphate (Kpomblekou and Tabatabai (1994). In contrast to nitrogen little attention has been given to the

inorganic transformation of P, but there is some evidence for biologically catalyzed changes in the oxidation state of this element too (Willet, 1989).

Biological oxidation of reduced P compound was demonstrated by Alexander, (1961) who noted that phosphite added to soil disappear with a corresponding increase in the concentration of phosphate, reaction 5.

HPO_3" ---> HPCV⁻ (5)

The conversion is brought about microbiologically since the reaction is eliminated upon the addition of a biological inhibitor such as toluene. A number of heterothrophic bacteria, fungi, and actinomycetes utilize phospohite within the cell to organic phosphate compounds. Bacteria utilize phosphite as sole P source in culture media and oxidize the phosphite within the cell to organic phosphate compounds. Bacteria utilize Phosphate in preference to phosphite so that, in media containing both anions, the former disappears first. There is no evidence that the oxidation is capable of providing energy for the development of chemoautothrophic bacteria.

The possibility of the reverse process, a reductive pathway, has received somewhat more attention. When a soil sample is incubated anaerobically in a mannitol-NH_4H_2PO4 medium, the phosphate disappears rapidly. This decrease is not a result of assimilation, which can only account for a small proportion of the loss. Phosphate apparently is reduced to phosphite and hypophosphite, and phosphine may possibly be evolved in the transformation (Eivazi and Tabatabai, 1977).

H3PO4 — 2H—>H_3PO_4—2H--->H_3PO_2"-> 4 H "^ PH_3 (2)

In the presence of nitrate or sulfate, phosphate reduction is retarded since the nitrate and sulfate seems to be more readily utilized as electron acceptors. More over, pure culture of *Clostridium butyricum* and *Escherichia coli* form phosphite and hypophosphite from orthophosphate (Rao, 1982). The process seems analogous biochemically to denitrification or to bacterial conversion of sulfate to sulfide. It is unlikely that the reduction takes place in well-aerated environments. The mineralization and immobilization of P are related to the analogous reaction of N (Dick and Tabatabai, 1987). As a rule, P release is more rapid under condition favoring ammonification (Tisdale *et al.,* 1985). Thus, highly significant correlation is observed between the rate of N and P conversion to inorganic form, the nitrogen mineralized being from 8 to 15 times amount of P made available. There is also correlation between and P mineralization, a ratio of C:N:P mineralized microbiologically at the equilibrium condition is similar to the ratios of these there element in humus (Garcia-Gil et al., 2000).

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