Menara Perkebunan, 2000 (68),2

Solubilization of insoluble phosphates by *Aspergillus niger*

Pelarutan fosfat sukar larut oleh Aspergillus niger

LAKSMITA P. SANTI[1)], D. H. GOENADI[1)], SISWANTO[1)],
I. SAILAH[2)] & ISROI[3)]

[1)] Biotechnology Research Unit for Estate Crops, Bogor 16151, Indonesia
[2)] Bogor Agricultural University, Bogor, Indonesia
[3)] Graduate School, Bogor Agricultural University, Bogor, Indonesia

Ringkasan

Penggunaan langsung fosfat alam (FA) ke dalam tanah sebagai sumber pupuk P telah dilakukan selama bertahun-tahun melalui beberapa macam cara penggunaan. Kualitas FA di Indonesia umumnya rendah dan ketersediaan bahan baku yang berkualitas untuk produksi pupuk fosfat terlarut relatif terbatas. Beberapa mikroba asal tanah yang dapat melarutkan fosfat anorganik telah banyak dilaporkan. Namun, informasi yang tersedia tentang mekanisme pelarutan P dari FA lokal asal Indonesia dan P anorganik oleh Aspergillus niger *BCC F194 belum banyak diteliti. Satu seri penelitian laboratorium telah dilaksanakan untuk mengetahui kemampuan* A. niger *BCC F194 melarutkan P. Evaluasi agronomi FA lokal (FA Cileungsi dan Madura) di rumah kaca juga telah dilakukan.* A. niger *BCC F194 dapat melarutkan sumber P sukar larut, yaitu FA Cileungsi dan Madura, serta senyawa $Ca_3(PO_4)_2$ dan $AlPO_4$. Kelarutan P anorganik tersebut berhubungan dengan peningkatan aktivitas proton (H*) yang menyebabkan penurunan pH medium dan produksi asam organik. Asam organik utama yang dihasilkan oleh* A. niger *BCC F194 dalam medium cair Pikovskaya yang dimodifikasi adalah asam oksalat (3.75 mM), asam sitrat (2.0 mM), dan asam glukonat (0.9 mM). Kelarutan FA Cileungsi lebih besar dibandingkan dengan FA Madura, dan kelarutan $Ca_3(PO_4)_2$ lebih besar dibandingkan kelarutan $AlPO_4$. Tidak ada korelasi antara kelarutan P anorganik dengan aktivitas enzim fosfatase, walaupun aktivitas enzim fosfatase cukup tinggi terdeteksi dalam medium. Satu formula biosuperfosfat telah berhasil dirakit dengan mereaksikan FA lokal dengan supernatan kultur cair (SKC) pengganti asam sulfat. Hasil percobaan pada bibit kakao, karet dan kelapa sawit di rumah kaca menunjukkan bahwa prototipe pupuk biosuperfosfat dengan bahan baku FA Cileungsi dan Madura bentuk granul maupun serbuk, memiliki nilai efektivitas agronomi yang relatif menyamai SP-konvensional.*

Summary

The direct application of rock phosphate (RP) to soils as a source of phosphorus (P) fertilizer has been employed with varying popular methods over the years. The RP in Indonesia has low quality for plant fertilization and the availability of the raw material with good quality for production of soluble phosphate fertilizers is limited. Many common soil microbes that can dissolve insoluble inorganic phosphate have been extensively studied. However, there is little information on mechanism of P-solubilization from local RP of Indonesia and inorganic P by *Aspergillus niger* BCC F194 isolated from tropical acid soils. A laboratory study was conducted to determine the ability of phosphate solubilization *A. niger* BCC F194. Agronomic evaluation of bioactivated local RP, *i.e*. Cileungsi and Madura phosphate rocks (CRP and MRP) for direct application in greenhouse experiment was also conducted. *A. niger* BCC F194 was able in solubilizing different types of hardly-soluble phosphates, *i.e*. CRP and MRP, $Ca_3(PO_4)_2$, and $AlPO_4$ compounds. Inorganic P solubilization was directly correlated to the organic acid production and increasing proton (H+) activities causing pH to decrease and production of organic acid. The major acidic metabolites produced by *A. niger* BCC F 194 in modified liquid culture Pikovskaya medium were oxalic acid (3.75 mM), citric acid (2.0 mM), and gluconic acid (0.9 mM). The solubilization of Cileungsi RP was higher than that of Madura RP, and the solubilization of $Ca_3(PO_4)_2$ was better than that of $AlPO_4$. No correlation between solubilization of inorganic P and enzyme activities, although high activities of phosphatase enzyme were detectable in the medium. A biosuperphosphate formula had been constructed by reacting local RP with liquid culture supernatant (LCS) replacing sulfuric acid. Results of cocoa, rubber, and oil palm seedlings experiments in greenhouse indicate that both granular and powder biosuperphosphate prototypes commonly had a comparable relative agronomic effectiveness value to that of the conventional SP.

[*Keywords* : P solubilization, *Aspergillus niger*, rock phosphate]

Introduction

Most rock phosphate deposits found in the world are classified as low reactive RPs and therefore, it cannot be used successfully as phosphorus (P) sources for crop production. The composition of these rock phosphates varies from one deposit to another. Most of the world rock phosphates are of sedimentary origin. Large rock phosphate deposits estimated 1-2 million tonnes, are distributed in different parts of Indonesia (Moersidi, 1999). Since these deposits are low in phosphorous content (<25% P_2O_5) and contain some impurities like oxides of Si, Fe, Al, and Ca, they are unsuitable for the manufacture of superphosphate (Narsian *et al*., 1993). The conventional method for enhancing the rock phosphate availability is to increase its solubility by treating with inorganic acids, mainly sulphuric acid and phosphoric acid; but, this approach is not applicable because of high capital production (Hammond *et al*., 1989, Goenadi *et al*., 2000). A very attractive approach for rock phosphate solubilization is the application of microbes capable of excreting organic acids (Gerke, 1992).

Many soil microbes are known to solubilize insoluble forms of inorganic phosphatic compounds. *In vitro* studies with microbial isolates from soil indicated that fungi were more efficient in the solubilization of inorganic phosphate as compared to bacteria (Thomas *et al*., 1985; Nahas 1996; Goenadi *et al*., 1999). Filamentous fungi are widely used as producers of organic acids, particularly *A. niger* and some *Penicillium* sp, which have been tested in fermentation systems or inoculated directly into soil in order to solubilize rock phosphate (Vassilev *et al*., 1997; Vasileva *et al.*, 1998).

Acidification of soil is a consequence of natural and anthropogenic processes (Ulrich & Sumner, 1991). One of the outstanding changes in soil due to acidification is the mobilization of Al^{3+} ions, which are toxic to plants on one hand and cause chemical fixation of plant-available P on the other hand. Hardly-soluble aluminum phosphates are formed and become the largest P-fraction in many acidic soils (McLean, 1976). $AlPO_4$, in the contrary to calsium phosphate, will never play an important role in ameliorating soils. Leaving that out of consideration, the importance of $AlPO_4$ solubilization for soil formation, mineral transformation and Al-toxicity is obvious (Illmer *et al*., 1995). In this investigation, pure insoluble inorganic phosphates *i.e.* aluminum phosphate ($AlPO_4$) and calcium phosphate ($Ca_3(PO_4)_2$) have been used for expressing phosphate solubilizing activity of *A. niger* BCC F194.

Natural RPs has been recognized as a valuable alternative source for P fertilizer, especially for acid soils. The economic value of the rocks increases considerably along with the increasing costs of SP production (Goenadi *et al*., 2000). Consequently, there is a growing interest in ways of manipulating such rock to obtain a more valuable product, *i.e*. partially acidulating RPs (Goenadi, 1990; Rajan and Ghani, 1997), reacting with synthetic organic acids (Sagoe *et al*., 1998) or natural organic acids (Singh & Amberger, 1998), and decreasing particle size (Babare *et al.*, 1997). Goenadi *et al*. (2000) reported that by using Liquid Culture Supernatant (LCS) instead of H_2SO_4 in superphosphate (SP) production and using both with lower H_3PO_4 concentration to bioactivated Morrocan RP as raw materials was found to increasing the solubility of P in 2% citric acid. Indications were obtained that LCS could replace H_2SO_4 in the production of SP , and believed to yield a more eco-friendly P fertilizer than conventional SP.

The objectives of this study were: (i) determining phosphate solubilizing (PS) activity by *A.niger* BCC F194 on four types of inorganic P compounds, (ii) effect of organic acids and phosphatase enzyme on RPs dissolution, and (iii) agronomic evaluation of bioactivated CRP and MRP for direct application in greenhouse experiment with cocoa, rubber, and oil palm seedlings as test crops.

Solubilation of insoluble phosphates by Aspergillus niger

Materials and Methods

Isolate

Aspergillus niger was isolated from Alfisols soils of Jeneponto, South Sulawesi, Indonesia (Goenadi *et al*., 1995). This soil was highly weathered developing over volcanic materials. Detailed characteristics of the soil are, 31% sand, 27% silts, and 42% clays; pH: 6.6 (H_2O), 5.3 (KCl); organic matter : 1.1% (C), 0.1% (total N); 105 ppm P, 35 me/100g K; and clay mineral of smectite and kaolinite. The isolate was lodged in Balitvet Culture Collection no. BCC F 194. Isolate was grown on Pikovskaya medium in petri dishes as a source of inoculum.

Phosphate solubilizing (PS) activity

Phosphate solubilizing activity was assayed in 50 mL aliquots of standard Pikovskaya's broth and modified Pikovskaya's broth prepared by replacing $Ca_3(PO_4)_2$ (19.9% P) with inorganic phosphate i.e. $AlPO_4$ (50.8% P) and different rock phosphates, i.e. Cileungsi (8.9% P) and Madura (6.9% P). Cileungsi rock phosphate (CRP) and Madura rock phosphate (MRP) were ground (200 mesh) and dry-oven. For this purpose, P sources were sterilized separately and then mixed with the sterile Pikovskaya medium.
Two pieces of inocula (Ø 8 mm) were inoculated into Pikovskaya liquid medium containing 0.125 %(w/v) respective phosphorus
sources, incubated on a mechanical shaker at 100 rpm, 28^{o}C for nine dyas. The dissolved P was then determined by applying the molybdenum-blue method of Olsen & Sommers (1982). Absorbance was measured using a Spectronic 21 spectrophotometer at 693 nm.

Solubilization of RPs in Pikovskaya medium

Modified Pikovskaya liquid medium supplemented with 0; 0.125; 0.25; 0.50; and 1.0 % (w/v) CRP and MRP respectively was used. Inoculation was performed by transferring two
pieces of inocula (Ø 8 mm) on to 100 mL Erlenmeyer flask containing 50 mL culture medium shaker at 100 rpm (28^{o}C) for nine days. The cultures were incubated on a mechanical.

The growth of the fungus was measured in standard procedure by drying the decanted mycelium in an oven at 70^{o}C to constant weight at the end of incubation. Phosphorus solubilizing ability was determined by using the molybdenum- blue method of Olsen & Sommers (1982). pH of the filtrates was measured with Methrom pH meter and organic acids concentration in the culture determined by using HPLC (0.01 N H_2SO_4 mobile phase, 210 nm UV detector, 0.5 mL.min^{-1} flowrate, at 50^{o}C) (Cunningham & Kuiack, 1992).

Effect of organic acids on RPs dissolution

This experiment was carried out to clarify the relative strength of different types of organic acids in solubilizing P from the CRP and MRP by using a method of Illmer *et al*. (1995). Citrate, oxalate and gluconate were added separately in different concentration (0; 0.05; 0.50; 1.0; 3.0 and 6.0 mM) to 50 mL Pikovskaya sterile medium containing 0.125% (w/v) CRP or MRP, then incubated for seven days on a mechanical shaker, at 100 rpm (28 ^oC). P-concentration in supernatant solutions was determined using the method of Olsen & Sommers (1982).

Effect of phosphatase enzyme on RPs and inorganic P dissolution

The isolate of A. niger BCC F 194 was grown in 50 mL modified Pikovskaya liquid medium with various levels of CRP or MRP (0; 0.125; 0.25;0.50 and 1.0%(w/v)). The P sources were sterilized separately and then mixed with the sterile Pikovskaya medium. The cultures were incubated for nine days on a mechanical shaker 100 rpm, (28^o C) for the production of extracellular phosphatase. Correlation between activity of phosphatase (Souciet *et al*., 1980) and P-solubilizing was determined. One enzyme unit is the amount which catalyses the hydrolysis of 1 μmol of pNPP per min. under the experimental condition.
Inactivation of phophatase in liquid culture supernatant (LCS) of *A. niger* BCC F194 was studied at temperature of 40, 50, 60, 70, 80, and 90 ^{o} C. Further, 50 mL LCS of *A.niger* BCC F194 contained inactivated phosphatase was added with

0.125% (w/v) $AlPO_4$ sterile and incubated on mechanical shaker at 100 rpm, 28 ^{o} C for 24 h. Activity of phosphatase enzyme (Souciet et al., 1980), P-solubilization, and concentration of organic acid (mainly citric acid) were determined.

Agronomic evaluation of bioactivated CRP and MRP

Bioactivation was conducted by reacting 8.5 mL LCS with the highest contents of organic acids (mainly citric acid from *A.niger* BCC F194 isolate) and 28 mL H_3PO_4 52% (v/v) on 55 g RPs (200 mesh) (Goenadi *et al*., 2000). The most efficient bioactivation of CRP and MRP were evaluated on the basis of soluble P contents in water and citric acid, as well as perchlorate-extractable P content (SII 0029-73, 1984). Effectiveness of the both granular and powder biosuperphosphate prototypes in substituting conventional P fertilizer, *i.e*. SP-36, was determined on the basis of relative agronomic effectiveness (RAE) (Mackay *et al*., 1984) in a completely random design experiment with four levels of P dosages, *i.e*. 0, 50, 100 and 150% to that of recommended SP-36 dosage. The RAE (%) was evaluated on the basis of seedling's dry weight that calculated from a greenhouse experiment using cocoa, rubber, and oil palm seedlings grown on Cikopomayak Ultisols, West Java (pH 4.7, 18.1 ppm P_2O_5 (Olsen test), total N 0.10%, organic matter 2.9 % (C), and 0.1 % (N)).

Results and Discussion

Phosphate solubilizing (PS) activity

A. niger BCC F194 could solubilize $Ca_3(PO_4)_2$, CRP, and MRP, but showed poor in $AlPO_4$ solubilization in nine days incubation period. The amount of P solubilized by *A. niger* BCC F194 was directly related to the decrease in pH of the medium, except $AlPO_4$ treatment. These phenomena suggested that PS activity depends on the types and constanta solubility product (K*sp*) of insoluble phosphate (K*sp* for $AlPO_4$ ~ 10^{-30} and $Ca_3(PO_4)_2$ ~ 2.0 x 10^{-20}) - as supposed by many investigators (Mc Lean, 1976; Narsian *et al*. 1993; Illmer *et al*., 1995; Nahas, 1996).

Solubilization of RPs in Pikovskaya medium

Employing a modified Pikovskaya medium, *A. niger* BCC F194 produced oxalic acid (3.75 mM), citric acid (2.0 mM), and gluconic acid (0.9 mM) as the main organic acids from MRP and CRP as P source of

Pikovskaya medium at 0 – 1.0% (w/v) level. Figure 1. shows the relationship among P-solubilizing ability and pH of the medium, or organic acid produced in a modified Pikovskaya medium.

Regression analysis indicates that P-solubilization was highly positive correlated with organic acid concentration (r_{MRP}=0.92** and r_{CRP}=0.85**) and negative correlated with the pH of the medium (r_{MRP}=-0.87* and r_{CRP}=-0.99**) (Figure 1). Organic acid concentrations were also strongly negative correlated with the pH of the medium (r_{MRP} =-0.99** and r_{CRP}=-0.87*).

Effect of organic acids on RPs dissolution

Production of organic acids is an important mechanism for solubilizing inorganic phosphate, although not the only possible one. Gluconic acid concentrations in Pikovskaya medium contained CRP and MRP as P source were comparatively constant, whereas the increased citrate and oxalate concentrations were apparently related to the increase in CRP and MRP concentrations in solution (Figure 2). It indicates that citrate and oxalate have strong influences to the solubilization of P-RPs, r_{CRP}=0.96**, and r_{MRP}=0.99** (citric acid) and r_{CRP}=0.93** and r_{MRP}= 0.86* (oxalic acid). On the other hand, no correlation between gluconic acid concentration and dissolution of CRP and MRP ($r_{CRP}=0.6^{ns}$ and r_{MRP}= 0.1^{ns}) were found. These phenomena lead to the assumption that citric acid and oxalic acid produced by *A. niger* BCC F194 isolate are responsible for lowering the pH of medium providing protons (H^+) to increase the P-RP solubilization. In this case, there was relationship between the pKa values of the acids and the amounts of P released. Citric acid has a higher dissociation constant (pKa=3.14) than oxalic acid

Table 1. Solubilization of $Ca_3(PO_4)_2$, $AlPO_4$, Cileungsi rock phosphate (CRP), and Madura rock phosphate (MRP) by *Aspergillus niger* BCC F194 in nine days incubation.

P-Sources	PS ability (%)	Initial pH	Final pH
$Ca_3(PO_4)_2$	65.0	7.3	4.3
$AlPO_4$	7.8	6.0	2.8
Cileungsi rock phosphate	95.0	6.8	4.2
Madura rock phosphate	57.6	7.6	6.3

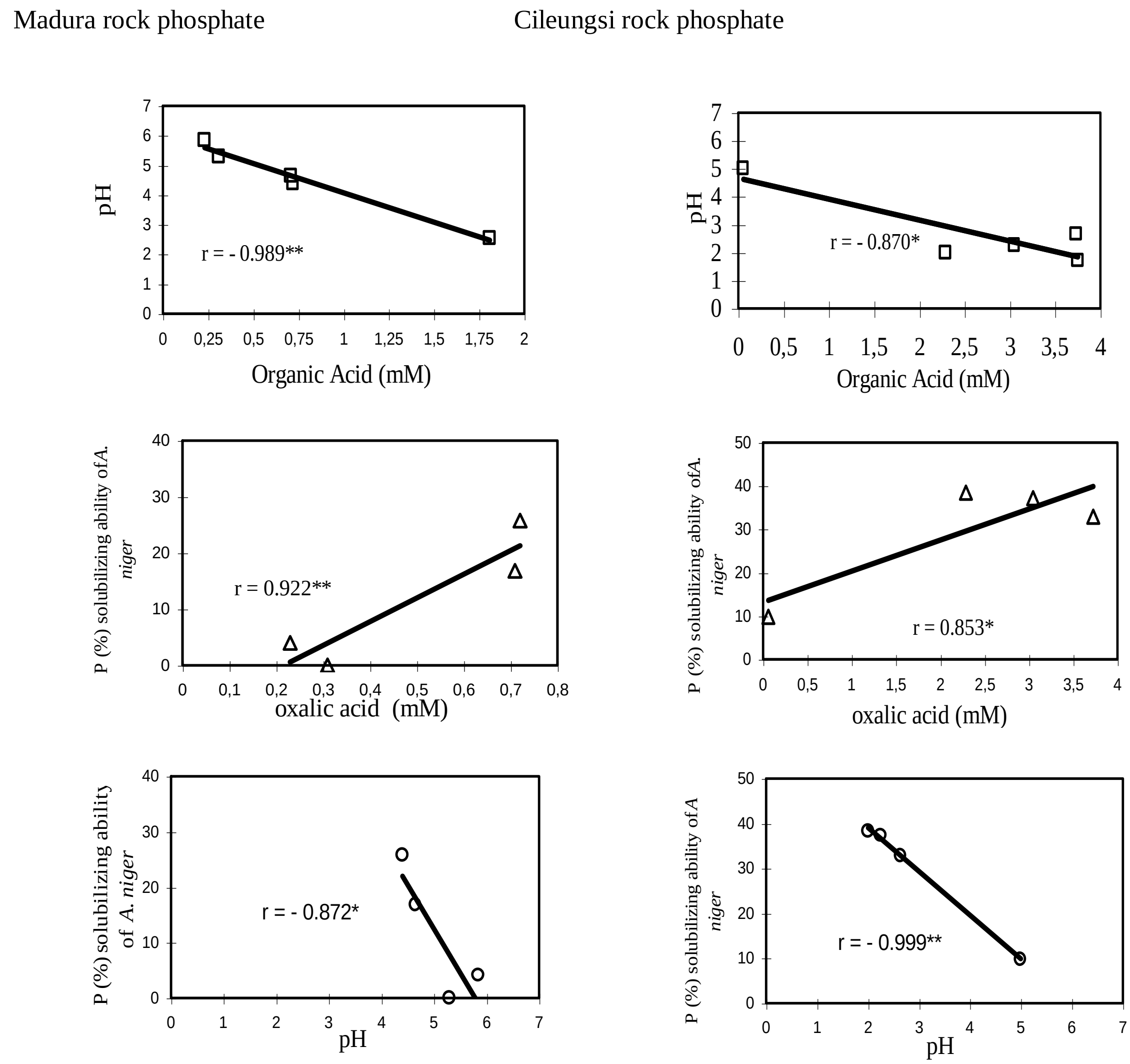


Figure 1. The correlation between pH and organic acid production (top), P - solubilizing ability of *A. niger* BCC F194 and oxalic acid production (middle), and P- solubilizing ability and pH (bottom) with rock phosphate from Madura and from Cileungsi as P sources in Pikovskaya medium.

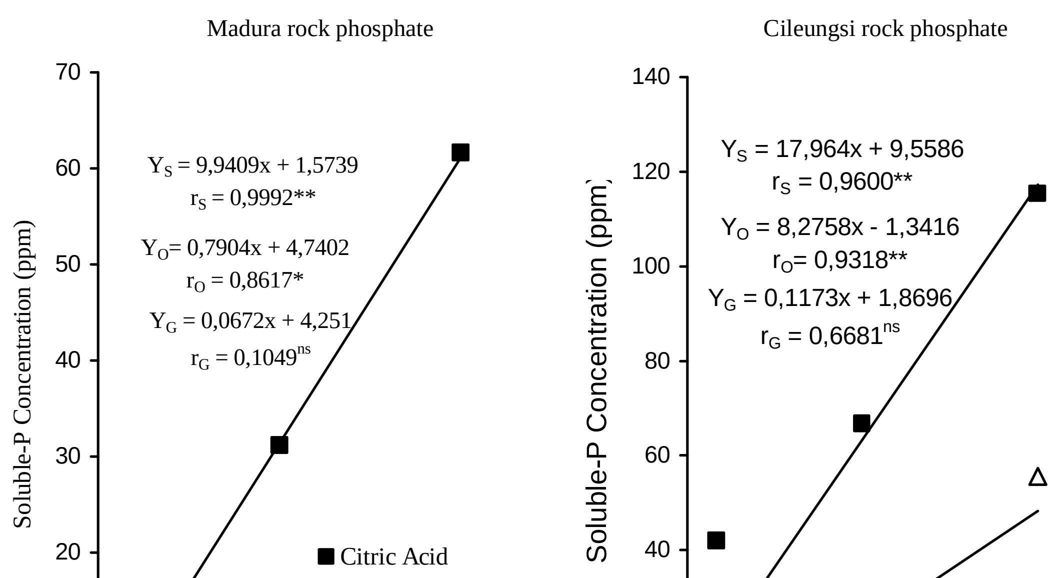
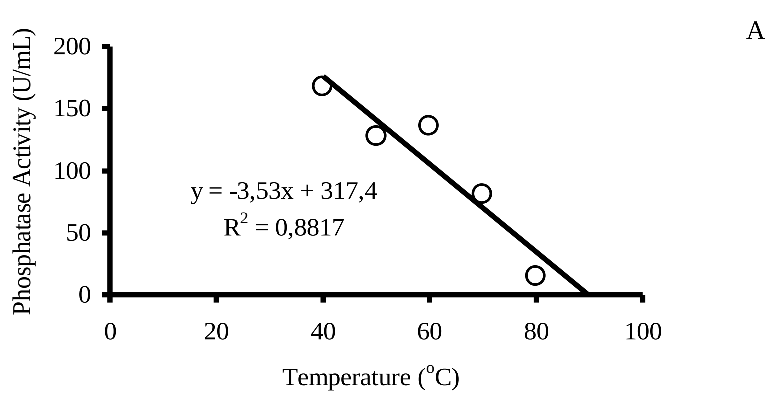


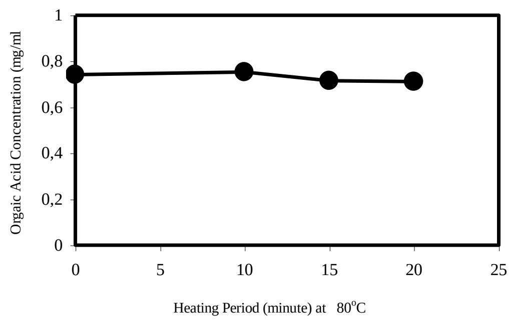
Figure 2. Relationship between concentration of selected organic acids (citric, oxalic, and gluconic) and solubilization of rock phosphate from Madura and Cileungsi in modified Pikovskaya medium.

(pKa = 1.25). Pohlman & McColl (1986) reported that several factors are important in determining the degree or rate of dissolution of RPs *i.e.* : (i) rate of diffusion of organic acid from bulk solution and diffusion of products from the site of reactivity, (ii) contact time between the organic acids and mineral surface, (iii) degree of dissociation of organic acids, (iv) type and position of functional groups, and (v) chemical affinities of chelating agents for the metals.

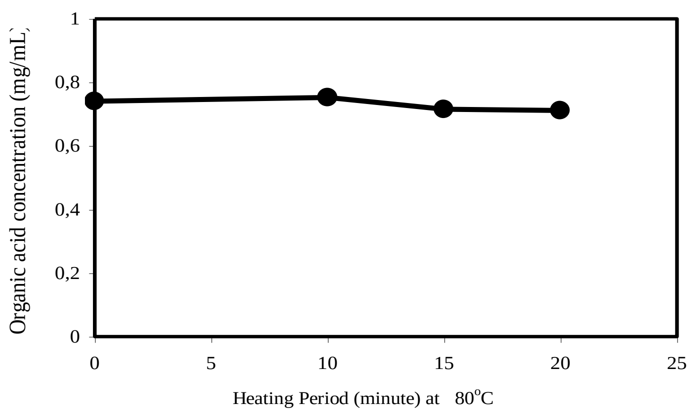
Effect of phosphatase enzyme on RPs and inorganic P dissolution

Phosphate solubilization mediated by phosphatase enzyme is believed to be taken place for organic P sources (Bishop *et al*., 1994). There was no correlation between activity of phosphatase enzyme and P solubilization (r_{CRP}=0.39^{ns} and r_{MRP}=0.22^{ns}), as well as with P concentration in (r_{MRP}=0.39^{ns} and r_{CRP}=0.23^{ns}). The phosphatase activity Pikovsakaya medium decreased at 80^{o}C (15 U/mL) and 90^{o}C (5.8 U/mL) (Figure 3A). Complete denaturation of protein by heating process possibly occurred at 80^{o}C, therefore phosphatase enzyme was inactivated. The correlation between phosphatase activity and Al PO_4 solubilization indicates that no significant of P-solubilizing value in liquid culture supernatant of *A. niger* BCC F 194 unheated (UH) and heated (H) at 80^{o} C, although the phosphatase activity was different, i.e. 127.3 U/mL (UH) and





B



C

Figure 3. Effect of temperature (A), and heating period (minute) at 80°C on activity of phosphatase enzyme (B), and effect of heating period at 80° C on organic acid (citric acid) concentration (C).
Santi et al.

Table 1. Relative agronomic effectiveness of constructed effect of biosuperphosphate (Spab) of cocoa, rubber, and oil palm seedlings dry weight.

Types of biosuper-phosphate	Form	Rate	Relative Agronomic Effectiveness (%)		
			cocoa seedlings	rubber seedlings	oil palm seedlings
Cileungsi	granule	50	62.5 b*)	65.8 c	120.2 abc
Cileungsi	granule	100	96.1 ab	96.9 bc	101.3 abc
Cileungsi	granule	150	89.2 ab	166.9 a	77.9 c
Cileungsi	powder	50	80.5 ab	146.9 ab	114.2 abc

Cileungsi	powder	100	80.1 ab	137.5 ab	93.3 abc
Cileungsi	powder	150	59.7 b	120.9 abc	92.5 abc
Madura	granule	50	69.5 b	104.2 abc	166.9 a
Madura	granule	100	85.5 ab	103.9 abc	127.9 abc
Madura	granule	150	115.2 a	150.9 ab	153.9 ab
Madura	powder	50	93.1 ab	61.1 c	83.7 bc
Madura	powder	100	113.8 a	97.6 bc	97.9 abc
Madura	powder	150	78.6 ab	99.7 bc	110.3 abc
Standard (SP-36)		100	100.0 ab	100.0 bc	100.0 abc

Note: *) *Figures in each column followed by the same letter (s) are not significantly different (P<0.05) according to Duncan's test.*

7
.9 U/mL (H), respectively (Figure 3B). On the other hand, the citric acid concentration was relatively stable at 0-20 minute of heating periods (Figure 3C). These results were in agree with assumption that phosphate solubilization mediated by phosphatase enzyme is taken place for organic P sources (Traina *et al*., 1986; Bishop *et al*., 1994; Sigh & Amberger, 1998).

Agronomic evaluation of bioactivated CRP and MRP for direct application in greenhouse experiment

Application of bioactivated CRP and MRP resulted in significant increase of leaf number and height of the three-month old cocoa and oil palm seedlings compared to the control pot. For rubber seedlings, significant responses have been observed starting at four months after treatment (P<0.05). There were no significant differences between conventional SP and biosuperphosphate (SPab) applied on height, leaf number, girth, and dry weights of cocoa, rubber, and oil palm seedling (unpublished data). At the end of the experiment (four months after treatment for cocoa and rubber, and six months for oil palm seedling), CRP and MRP originating SPab in granular and powder form applied at 100% rate equivalent to standard SP application indicated a higher value of relative agronomic effectiveness (RAE) (Table 1).

Conclusions

A. niger BCC F194 performed high abilities in solubilizing inorganic phosphate (>50%), i.e. $Ca_3(PO_4)_2$, CRP, and MRP, but not for $AlPO_4$. Citric and oxalic acids were important components responsible for phosphate solubilization by *A. niger* BCC F194. Oxalic acid was the main organic acid produced by *A. niger* BCC F194 in modified Pikovskaya medium, for nine days incubation at 100 rpm, 28^{o}C. Citrate and oxalate had strong influences to the solubilization of CRP and MRP. There was no correlation between phosphatase activity and solubilization of inorganic phosphate. Results of cocoa, rubber, and oil palm seedlings experiments in greenhouse showed that the prototype products from bioactivation (biosuperphosphate) had a comparable relative agronomic effectiveness value to that of the conventional SP (SP-36).

Acknowledgment

The authors wish to thank the Riset Unggulan Terpadu VII Project Management of the Ministry of Research and Technology for financial support (Contract No.: 17/SPK/RUT/BPPT/IV/2000).

References

Babare, A.M., P.W.G. Sale, N. Fleming, D.L. Garden, & D. Johnson (1997). The agronomic effectiveness of reactive phosphate rocks 5. The effect of particle size of a moderately reactive phosphate rock. *Aus. J. Exp. Agric*., **37**, 969-984.

Bishop, M.I., A.C. Chang, & R.W.K. Lee (1994). Enzymatic mineralization of organic phosphorus in a volcanic soil in Chile. *Soil Biol. Biochem.*, **157**, 238-243.

Cunningham, J.E., & C. Kuiack (1992). Productions of citric and oxalic acids and solubilizations of calsium phosphate by *Penicillium bilaji*. *App.Env. Microbiol*. **58**(5), 1452-1458.

Gerke, L. (1992). Phosphate, aluminium, and iron in solution of three different soils in relation to varying concentration of citric acid. *J. Plant Nutr. Soil Sc.,* **155**, 339-343.

Goenadi, D.H. (1990). Effect of acidulation on the mineralogical characteristics of a commercial phosphate rock. *Ind. J. Trop. Agric*., **2**, 1-5.

Goenadi, D.H., R. Saraswati, N.N.Nganro, & J.A.S. Adiningsih (1995). Mikroba pelarut hara dan pemantap agregat dari beberapa tanah tropika basah. *Menara Perkebunan*, **63**(2), 60-66.

Goenadi, D.H., R.A. Pasaribu, Isroi, H. Hartono, & R. Misman (1999). Phosphate-solubilizing fungi isolated from tropical forest soils *Menara Perkebunan,* **67**(1), 40-51.

Goenadi, D.H., Siswanto & Y. Sugiarto (2000). Bioactivation of poorly soluble phosphate rocks with a P solubilizing fungus. *Soil. Sci. Soc.Am.J*., **64**, 927-932

Hammond, L. L., S.H Chien & A.U. Mokwunye (1989). Agronomic value of unacidulated and partially acidulated phosphate rocks indigenous to the tropics. *Adv. Agr.*, **40**, 89-140.

Illmer. P., A. Barbato, & F. Schinner (1995). Solubilization of hardly-soluble AlPO4 with P-solubilizing microorganisms. *Soil Biol. Biochem.,* **27** (3), 265-270.

Mackay, A.D., J.K. Syers & P.E.H. Gregg (1984). Ability of chemical extraction procedures to assess the agronomic effectiveness of phosphate rock materials. *New Zealand J. Agric. Res*., **27**, 219-230.

McLean E.O. (1976). Chemistry of soil aluminium. *Communications in Soil Sci. & Plant Anal.*, **7**, 619-636.

Moersidi (1999). Fosfat Alam sebagai Bahan Baku dan Pupuk Fosfat. Bogor, Pusat Penelitian Tanah dan Agroklimat, pp 82.

Narsian V., J. Thakkar, & H.H. Patel (1993). Solubilization of natural rock phosphates and pure insoluble inorganic phosphates by *Aspergillus awamori*. *Ind. J. Exp. Biol*., **31**, 747-749.

Olsen, S.R., & L.E. Sommers (1982). Phosphorus. *in* A.L.Page, R.H. Miller, & D.R. Keeney (Eds.) *Methods of Soil Analysis. Agronomy series*, No. 9. Madison, American Society of Agronomy, p. 403-430.

Pohlman, A.A., & J.G. McColl (1986). Kinetics of metal dissolution from forest soils by soluble organic acids. *J. Environ. Qual*., **15,** 86-92.

Rajan, S.S.S. & A. Ghani (1997). Differential influence of soil pH on the availability of partially sulphuric and phosphoric acidulated phosphate rocks. 2. Chemical and scanning electron microscopic studies. *Nut. Cyc. Agroecosyst*., **48** (3), 171-178.

Sagoe, C.I., T. Ando, K. Kouno, & T. Nagaoka. (1996). Response of Italian ryegrass to phosphorus in organic – acid treated phosphate rocks. *J. Fac. Appl. Biol. Sci*. **35**, 199-209.

Singh, C.P. & A. Amberger (1998). Organic acids and phosphorus solubilization in straw composted with rock phosphate. *Bioresource Technol.,* **63**(1), 13-16.

Souciet, G., Attias. J & J. d' Auzac (1980). A neutral cytoplasmic phosphatase from the latex of *Hevea brasiliensis. Phytochem.*, **19**, 2099-2102.

Thomas, G.V., M.V. Shantaram, & N. Saraswathy (1985). Occurrence and activity of phosphate-solubilizing fungi from coconut plantation soils. *Plant & Soil*, **87**, 357-364.

Traina, S.J, G. Sposito, D. Hesterberg, & U. Kafkafi (1986). Effects of pH and organic acids on orthophosphate solubility in acidic, montmorillonitic soil. Soil *Sci.Soc.Am.J*., **50**, 45-51.

Ulrich B. & M.E. Sumner (1991). *Soil Acidity*. Berlin., Springer-Verlag.

Vassilev, N., M. Vassileva, & R. Azcon (1997). Solubilization of rock phosphate by

immobilized *Aspergillus niger*. *Bioresource Tehnol.,* **59**, 1-4.

Vassileva, M., R. Azcon, J. Barea, and N. Vassilev (1998). Application of an encapsulated filamentous fungi in solubilization of inorganic phosphate. *J. Biotechnol.,* **63** (1), 67-72.