Bioactivation of Poorly Soluble Phosphate Rocks with a Phosphorus-Solubilizing Fungus

Didiek H. Goenadi,* Siswanto, and Yudho Sugianto

ABSTRACT

Many studies have demonstrated ineffectiveness of finely ground phosphate rock (PR) use due to the low solubility of its P contents. This study was conducted to develop a simple, effective, and environmentally sound process to improve P availability of PR to crops by using a phosphorus-solubilizing fungus (PSF), Aspergillus niger BCC F.194, isolated from tropical acid soils. The optimum incubation period and the optimum level of PR were determined. The P-solubilizing effect of the supernatant of 9-d-old liquid culture supernatant (LCS) of the fungus was also determined by reacting it at various concentrations with Moroccan phosphate rock (MPR). The inoculation of the growth media with the PSF A. niger resulted in the highest P solubility of the rock after 9 d of culturing at 2.5 g L⁻¹ MPR. Up to a certain degree, direct inoculation of fungal biomass and its LCS onto MPR caused a remarkable increase in 2% citric acid–soluble P, but not in water-soluble P content. The possibilities of using the LCS instead of H₂SO₄ in superphosphate (SP) production and using both with lower H₃PO₄ concentrations were investigated with MPR as raw materials. Replacement of H₂SO₄ by the LCS in the SP production process yielded a comparable 2% citric acid–soluble P content. Combining the LCS and H₂SO₄ reduced the consumption of H₃PO₄ that occurs in standard SP production. This LCS technique provides a practical means for an effective bioactivation of PR intended for both as a P fertilizer and a raw material of the SP.

Natural PRs have 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. Consequently, there is a growing interest in ways of manipulating such rock to obtain a more valuable product. Common efforts include the use of chemical-physical means, that is, partially acidulating PRs (Hammond et al., 1986; Goenadi, 1990; Lewis et al., 1997; Rajan and Ghani, 1997), reacting with synthetic organic acids (Sagoe et al., 1998) and/or natural organic acids (Singh and Amberger, 1998a, 1998b), and decreasing particle size (Babare et al., 1997). However, Simpson et al. (1997) reported that a reactive North Carolina phosphate rock was not an economically feasible fertilizer to apply annually under New Zealand conditions: that is, (i) on nonleaching sites where the soil P status was low, (ii) in soils with a very high P sorption capacity, and (iii) where North Carolina PR dissolution was restricted by low rainfall or high pH.

On the other hand, Thien and Myers (1992) indicated that by increasing soil microbial activities, bioavailability of P in a bioactive soil was remarkably enhanced. Such a phenomenon inspires the application of a similar principle on the bioactivation of relatively nonreactive PR. The fact that certain soil microbes are capable of dissolving relatively insoluble phosphatic compounds (Asea et al., 1988; Nahas et al., 1990; Bojinova et al., 1997) has opened the possibility for inducing microbial solubilization of phosphates in soil. Many investigators believed that the phenomenon was closely related with the ability of the microbes in producing selected organic acids, and/or extracellular polysaccharides (Kucey, 1983; Illmer and Schinner, 1992; Goenadi et al., 1993; Goenadi et al., 1995; Omar, 1998; Kim et al., 1998).

Combined direct application of PR and phosphate-solubilizing microbes (PSM) has produced mixed results on plant growth responses, which were perhaps attributed to differences in microbial strains and/or soils being treated. Inoculation of the PSM onto PR or reacting the PR with a LCS may be considered a better means to overcome the low solubility problems of PR (Goenadi, 1996). Such an approach may eliminate factors inhibiting a successful interaction between PSM and PR under field conditions. This approach will also make the production of single SP possible without the use of chemical acidulation.

This paper presents results of laboratory studies with the objectives of: (i) determining the effect of various dosages of fungal inoculum and incubation periods on the solubilization of P from PR, (ii) developing an effective bioactivated PR and (iii) in producing biosuperphosphate. It is hypothesized that direct application of a PSF or its LCS on PR, replacing chemical acidulation, can provide a reactive and environmentally safe P fertilizer.

MATERIALS AND METHODS

Optimizing Phosphate Rock Solubilization at Varying Incubation Periods and Phosphate Rock Concentrations

A PSF, Aspergillus niger BCC F.194, originating from a highly weathered tropical soil (clayey, kaolinitic, isohyperthermic Typic Paleudults) (Goenadi et al., 1995) was used for this study. A loop of a 7-d-old agar (Oxoid L11) plate culture of the fungus was inoculated into a series of 250-mL Erlenmeyer flasks containing 100 mL of a modified Pikovskaya broth. The medium; originally consisting of glucose 10 g, Ca₃(PO₄)₂ 5g, NaCl 0.2 g, KCl 0.2 g, MgSO₄ · 7H₂O 0.1 g, MnSO₄ · 7H₂O, FeSO₄ · 7H₂O 0.0025 g, (NH₄)SO₄ 0.5 g, and yeast extract 0.5 g in a liter of aquadest; was modified by replacing tricalcium phosphate with MPR (Narsian et al., 1995). A perchlorate-extractable, 2% citric acid–extractable, and water-soluble P contents of the MPR were 139 g kg⁻¹, 34 g kg⁻¹, and 0.07 g kg⁻¹, respectively. The flasks were shaken at 100 rpm at room temperature for 9 d.

Abbreviations: LCS, liquid culture supernatant; MPR, Moroccan phosphate rock; PR, phosphate rock; PSF, phosphate-solubilizing fungus; PSM, phosphate-solubilizing microbes; SP, superphosphate.

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temperature (≈28°C). At 3, 6, 9, and 14 d after inoculation, a series of flasks was selected and the supernatant was collected by centrifugation at 7200 rpm for 20 min (Thomas et al., 1985; Narsian et al., 1993). Inorganic-P (P_i) content was extracted by reacting the extract with trichloroacetic acid, ferrosulfate, and molybdate solutions (Taussky and Shorr, 1953). Although this method of Taussky and Shorr (1953) was originally designed for applications in medical science, we used this method because it is equally sensitive to the molybdenum blue method by soil scientists (Olsen and Sommers, 1982), and because it has the added advantage of less sample volume and chemicals required and faster color establishment. Measurement of P in the extract was conducted spectrophotometrically.
Bioactivation of Moroccan Phosphate Rock by Direct Inoculation of the Phosphate-Solubilizing Fungus on Moroccan Phosphate Rock

One hundred grams of 80-mesh MPR in a 0.25-L polyethylene bag previously sterilized by gamma-rays at 27 kGray was inoculated with a 7-d-old PSF liquid culture at dosages of 0, 12.5, 25, 37.5, and 50 mL liquid culture kg$^{-1}$ MPR. Prior to inoculation, the fungus suspension was stirred gently to provide homogeneous mixture of spores and mycelial fragments. Inoculation was conducted aseptically by injecting the liquid culture onto the sterilized MPR. The inoculum consisted of $10^{14}$ cfu per mL. All samples were adjusted to 1:1 solid/liquid ratio by adding sterilized water to arrive at comparable conditions. The inoculated samples were then incubated at room temperature for 7 and 14 d. At the end of the incubation period, the respective samples were analyzed for water- and 2% citric acid–soluble P using procedures as outlined by Rund (1984). Fungal population was determined using serial dilution and plating methods on a Pikovskaya solid medium. Colonies with clear zones surrounding them were then considered as culturable PSF.

Bioactivation of Moroccan Phosphate Rock by Liquid Culture Supernatant of Phosphate-Solubilizing Fungus

Liquid culture supernatant was obtained from 9-d-old cultures of PSF on Pikovskaya broth containing 2.5 g L$^{-1}$ MPR. The culture broth was centrifuged at room temperature at 7200 rpm for 20 min to separate the fungal biomass and supernatant containing phosphate-dissolving organic acids. The fungal biomass was oven dried, whereas the supernatant was used for the MPR activation process. One hundred grams, nonsterile, 80-mesh MPR was reacted with 0, 125, 250, 500, and 1000 mL LCS kg$^{-1}$ in 250-mL Erlenmeyer flasks. The liquid/solid ratio was maintained at 1:1 (v/w) by making up the volume with sterile deionized water. Reaction was conducted by shaking the mixtures on a mechanical shaker at 100 rpm and at room temperature for 2 h, after which the mixture was oven dried overnight at 105°C, and ground to 80 mesh size. Water- and 2% citric acid–soluble P–P$_2$O$_5$ contents were determined by the methods previously mentioned. Similar experiments were conducted using H$_2$SO$_4$ and various concentrations of H$_3$PO$_4$, and by replacing H$_2$SO$_4$ with various concentrations of LCS. These experiments, simulating SP production by acidulation of MPR with H$_2$SO$_4$ and/or followed by H$_3$PO$_4$ enrichment (Young et al., 1985), were conducted by treating of 5.2 g of 80-mesh MPR pretreated with 0, 125, 250, 500, and 1000 mL kg$^{-1}$ of LCS as described previously. Each of the LCS-treated samples was then reacted with 1.55 mL H$_2$SO$_4$ (98%) and 3.25 mL H$_3$PO$_4$ adjusted at various concentrations, that is, 50, 100, 150, and 200 mL H$_3$PO$_4$ L$^{-1}$, by diluting H$_3$PO$_4$ (52%) in distilled water. The mixture was shaken at 100 rpm for 2 h, and dried. For the last experiment, 55 g of 100- and 200-mesh MPR were evenly handsprayed with 28 mL H$_3$PO$_4$ (52%) and cured for 2 h followed by four levels of LCS, that is, 0, 85, 170, and 255 mL kg$^{-1}$, handsprayed onto the H$_3$PO$_4$-treated MPR and cured for another 2 h. As a reference, 550 g of 100- and 200-mesh MPR were reacted with 170 mL H$_2$SO$_4$ (98%) and 280 mL H$_3$PO$_4$ (52%) only. The ratio between PR and acids used in the last experiment is standard procedure in the production of Indonesian SP (Indonesian National Standard #02–3769–1995) when the standard MPR is used. All samples were then analyzed for perchloric-acid-, water-, and 2% citric acid–soluble P contents (Rund, 1984). Two replicates were employed for all experiments.

Statistical Analysis

Statistical analysis was conducted using Duncan Multiple Range Test (DMRT) ($P = 0.05$) to determine significant dif-

Fig. 4. Improvement on P solubility of the Moroccan phosphate rock treated with liquid culture supernatant (LCS) for 2 h at varying concentration. PSF is phosphate-solubilizing fungus.
Table 1. Soluble P contents of phosphate-solubilizing fungus’s liquid culture supernatant (LCS) pretreated Morrocan phosphate rock at various concentrations of H$_3$PO$_4$ and addition of H$_2$SO$_4$ (98%).

<table>
<thead>
<tr>
<th>LCS concentration mL kg$^{-1}$</th>
<th>H$_3$PO$_4$ concentrations (mL L$^{-1}$)</th>
<th>2% Citric acid</th>
<th>H$_2$O</th>
<th>2% Citric acid</th>
<th>H$_2$O</th>
<th>2% Citric acid</th>
<th>H$_2$O</th>
<th>2% Citric acid</th>
<th>H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td></td>
<td>74.4c p*</td>
<td>71.8c s</td>
<td>108.1a q</td>
<td>104.6a t</td>
<td>108.9c q</td>
<td>106.1ab t</td>
<td>123.6b r</td>
<td>116.3d u</td>
</tr>
<tr>
<td>125</td>
<td></td>
<td>95.7b p</td>
<td>89.8a s</td>
<td>111.9a q</td>
<td>100.6a t</td>
<td>113.9ab q</td>
<td>103.9bc t</td>
<td>126.0ab r</td>
<td>118.2c u</td>
</tr>
<tr>
<td>250</td>
<td></td>
<td>102.9ab p</td>
<td>89.4a s</td>
<td>112.9a pq</td>
<td>98.9a t</td>
<td>119.3a q</td>
<td>109.3a u</td>
<td>125.5a q</td>
<td>120.2a v</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td>97.7ab p</td>
<td>83.6b s</td>
<td>109.1a pq</td>
<td>100.9a t</td>
<td>120.4a q</td>
<td>103.9bc t</td>
<td>126.9a q</td>
<td>119.1b u</td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td>108.3a p</td>
<td>91.7a s</td>
<td>107.4a p</td>
<td>104.9a t</td>
<td>107.7bc p</td>
<td>126.7a t</td>
<td>126.7a p</td>
<td>115.1e u</td>
</tr>
</tbody>
</table>

* Figures followed by similar letter(s) in the same column (a, b, .... , e) and in the same row (p, q, .... , u) of the corresponding parameter are not significantly different according to DMRT ($P < 0.05$).

The amounts of P released (Pi) into the 2.5 g MPR L$^{-1}$ containing culture medium by A. niger and pH dynamics during 14 d of incubation are presented in Fig. 1. As shown in the figure, phosphate solubilization increased quadratically reaching an optimum at about 9 d of incubation. During the 9 d of culturing differences in Pi were insignificant among the treatments of 2.5, 5.0, and 10.0 g MPR L$^{-1}$ growth medium. Many have suggested that increasing P concentration in the phosphate-solubilizing fungal containing medium was related to the secretion of organic-acid-types metabolites, which should correlate with the pH of the medium (Illmer and Schinner, 1992; Illmer et al., 1995; Narsian et al., 1995). However, they failed to establish a clearcut relationship between phosphate solubilization and pH. On the other hand, Kim et al. (1997, 1998) showed a strong relationship between a drop in the pH and a drastic increase in soluble phosphate concentration. Our data (Fig. 1) exhibit a considerably close relationship between pH and the amount of P released ($r = -0.69^*)$.

The A. niger used appears capable of producing some organic acids. High performance liquid chromatography analysis showed that the isolate produced about 0.8 $\mu$M citrate mL$^{-1}$ medium, 1.6 $\mu$M malate mL$^{-1}$ medium, 0.3 $\mu$M p-tartrate mL$^{-1}$ medium, and 0.1 $\mu$M p-malate mL$^{-1}$ medium, under the above-mentioned culture conditions. These acids, especially citrate and malate, were known to solubilize relatively insoluble P (Tisdale and Nelson, 1975; Singh and Amberger, 1998b) and synergistic effects of acids alone have been proposed by Parks et al. (1990). The above-mentioned amounts of organic acids secreted by the fungus were considerably lower than reported in other studies. Illmer et al. (1995) indicated the level of organic acids resulting in significant P dissolution were in the order of 3 to 30 $\mu$M mL$^{-1}$, distinctly below the efficiency of biotic leaching. These investigators assumed that the production of organic acids is an important mechanism for solubilizing relatively insoluble P, but not the only possible one. One possibility would be the release of H$^+$ from the cytoplasm to the outer surface which may happen in exchange for cation (especially NH$_4^+$) uptake or with the help of H$^+$ translocating ATPase which is located in the plasmalemma and uses the energy for ATP hydrolysis (Illmer and Schinner, 1992). Following this hypothesis, these authors assumed that PR would be solubilized directly at the cell surface. If this is the case, then the mycelial dry weight will correlate closely with the soluble P. Our observation on mycelial dry weight; that is, 0, 2.44, 2.86, 1.88, and 1.44 mg mL$^{-1}$ at 0, 3, 6, 9, and 14 d of culturing, respectively; indicated a correlation with the amount of soluble P ($r = 0.62^*$). This evidence leads to support of the Illmer and Schinner’s hypothesis (1992).

### Bioactivation Effectiveness of Morrocan Phosphate Rock by Direct Inoculation of Phosphate Solubilizing Fungus Biomass

The solubility of P as a result of inoculation of PSF biomass at different periods of incubation was slightly dependent upon the dosage of inoculum (Fig. 2). A significant increase in Pi soluble in 2% citric acid of inoculated MPR was obtained at 25 mL kg$^{-1}$ dosage after 14 d of incubation. However, no remarkable improvement was noticed in Pi solubility of treated MPR extracted by H$_2$O, although both water- and citric-acid-soluble P tended to increase with increasing period of incubation. The solubility of P in treated MPR (Fig. 2) seemed to be governed by the fungal population (Fig. 3). Increasing the dosage of the inoculum to 50-mL liquid culture kg$^{-1}$ MPR produced a significant linear increase of the population of the PSF after 14 d of inoculation. However, the increase of P soluble in citric acid (Fig. 2) was more closely related to the pattern of population (Fig. 3) at 7 d after inoculation ($r^2 = 0.96^{**}$) than that of 14 d after inoculation ($r^2 = 0.34^{**}$). This implies that during the first 7 d after inoculation, the fungus was growing actively, and hence, increased secretion of organic acids.

The production of organic acids will provide more protons for solubilizing the rock (Illmer et al., 1995). Although P solubilization continued in the second 7 d of incubation, this process was less dependent on the PSF population. The tendency of reduction in phosphate...
The LCS in combination with H$_2$SO$_4$ may to some extent solubilize P (55.9 g P kg$^{-1}$ MPR). We greatly appreciate the financial support provided by Petrokimia Gresik (Persero) under Research Collaboration Contract No. 105/03/01.02/46/SP/1997.

Table 2. Soluble P contents of 100- and 200-mesh Moroccan phosphate rocks (MPRs) pretreated with 280 mL kg$^{-1}$ H$_2$PO$_4$ (52%) affected by different type of activating agents.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Soluble P content</th>
<th>Perchloric acid-extractable P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H$_2$O</td>
<td>2% Citric acid</td>
</tr>
<tr>
<td>100-mesh MPR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>0.08g$^\dagger$</td>
<td>50.5f</td>
</tr>
<tr>
<td>170 mL H$_2$SO$_4$ (98%) kg$^{-1}$</td>
<td>121.2a</td>
<td>129.7bc</td>
</tr>
<tr>
<td>85 mL LCS kg$^{-1}$</td>
<td>81.0d</td>
<td>137.8b</td>
</tr>
<tr>
<td>170 mL LCS kg$^{-1}$</td>
<td>102.6b</td>
<td>147.9a</td>
</tr>
<tr>
<td>255 mL LCS kg$^{-1}$</td>
<td>52.6f</td>
<td>103.2d</td>
</tr>
<tr>
<td>200-mesh MPR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>1.0g</td>
<td>65.8e</td>
</tr>
<tr>
<td>170 mL H$_2$SO$_4$ (98%) kg$^{-1}$</td>
<td>127.7a</td>
<td>131.0bc</td>
</tr>
<tr>
<td>85 mL LCS kg$^{-1}$</td>
<td>93.2c</td>
<td>132.2bc</td>
</tr>
<tr>
<td>170 mL LCS kg$^{-1}$</td>
<td>83.7b</td>
<td>137.6b</td>
</tr>
<tr>
<td>255 mL LCS kg$^{-1}$</td>
<td>72.8e</td>
<td>126.6c</td>
</tr>
</tbody>
</table>

$^\dagger$ Figures followed by similar letter(s) in the same column are not significantly different according to DMRT ($P < 0.05$). $^\ddagger$ LCS, liquid culture supernatant.

Solubilizing activity on the second 7 d of incubation may be attributed to the availability of a soluble form of phosphate that has an inhibiting effect on further phosphate solubilization (Narsian et al., 1995). Another explanation of this is the formation of an organo-P compound induced by organic metabolites released, which, in turn, reduces the amount of available P (Illmer and Schinner, 1992). Considering the length of the incubation period (14 d) and the corresponding insignificant effects on increasing the soluble P contents, the inoculation of PSF's biomass on MPR appeared to be an impractical method for bioactivation of MPR at industrial scale.

The Use of Liquid Culture Supernatant in Bioactivation of Moroccan Phosphate Rock

The standard operational procedure in SP production includes reaction of 200-mesh MPR with H$_2$SO$_4$ (98%) and H$_2$PO$_4$ (52%) (Young et al., 1985). The mass composition of these materials were 52% MPR and 48% acids (15.5% H$_2$SO$_4$ + 32.5% H$_2$PO$_4$). However, some variations in the mass composition may occur at industrial practices depending on the grade of the rock and/or the acids. Assuming now that such an acidulation with H$_2$SO$_4$ and H$_2$PO$_4$ could be replaced by organic acids capable of dissolving P, the MPR was reacted in our experiments with varying concentrations of LCS. The results indicated that the LCS pretreated MPR has higher 2% citric acid- and water-soluble P contents as compared with the untreated MPR (Fig. 4). Although the increase in water soluble P due to LCS addition was twice that of untreated MPR, the values were practically low (0.16 g P kg$^{-1}$). The addition of 250 mL LCS kg$^{-1}$ to the MPR resulted in the highest values of both 2% citric acid–soluble P (55.9 g P kg$^{-1}$) and water-soluble P. Although these values were slightly lower than those of direct fungal biomass inoculation, the above technique seemed to be more efficient as less time consumed in the dissolution of MPR.

In order to achieve more effective P solubilization, the LCS pretreated MPR was reacted with H$_2$SO$_4$ (98%) and H$_2$PO$_4$ at selected concentrations. Another purpose of this experiment was to determine whether the use of the LCS in combination with H$_2$SO$_4$ may to some extent save the H$_2$PO$_4$ consumption that occurs in standard operational procedures for SP. The data presented in Table 1 indicated that the solubility of MPR reactivity was greatly improved by the latter process. The increase in soluble P contents was not only caused by acidulation with H$_2$SO$_4$, to some extent, but also to enrichment of P from H$_2$PO$_4$. Reacting a 500-mL LCS kg$^{-1}$ pretreated MPR with H$_2$SO$_4$ (98%) appears to reduce the required concentration of H$_2$PO$_4$, that is, 20% (Table 1) instead of 52% as in the conventional process.

Soluble P contents increased compared to control when using either PSF’s LCS or H$_2$SO$_4$ (98%) in combination with H$_2$PO$_4$ (52%), both in 100- and 200-mesh MPRs (Table 2). Although the water-soluble P contents of MPR treated with LCS–H$_2$PO$_4$ were significantly lower than that treated with H$_2$SO$_4$–H$_2$PO$_4$, the citric acid soluble P contents between the two treatments were similar. The addition of PSF’s LCS affected quadratically the soluble P contents of the MPR, and an optimum level based on citric acid–soluble P was obtained at approximately 148 and 170 mL LCS per 550 g 100- and 200- mesh MPR, respectively, pretreated with 280 mL H$_3$PO$_4$ (52%). The observed data of soluble P contents at different LCS volumes (n = 4, excluding H$_2$SO$_4$ treatment) were highly fit to the quadratic equation model developed with $R^2 = 0.99^{**}$ and $R^2_{\text{citric acid}} = 0.99^{**}$ for 100-mesh and $R^2 = 0.97^{**}$ and $R^2_{\text{citric acid}} = 0.91^{**}$ for 200-mesh H$_2$PO$_4$ pretreated MPR. It was obvious that a bioactivated SP was produced with characteristics close to those of conventional SP. However, the agronomic effectiveness of this product awaits further studies.

CONCLUSIONS

Bioactivation of poorly soluble PRs was achieved by using PSF, a technique potentially applicable to the activation of PR intended as a raw material in the production of SP fertilizers. The three approaches evaluated, including inoculation of PSF biomass to PR, reacting PSF’s LCS with PR, and the use of PSF’s LCS in place of conventional H$_2$SO$_4$ essential in the production of SP, indicated that PSF biomass inoculation method needed a considerably long period of incubation to increase the soluble P content of the rock. This phenomenon was attributed to the period needed for the PSF to grow and produce sufficient amount of phosphate-dissolving organic acids. Reacting the PSF’s LCS with PR yielded better results whereas the use of PSF’s LCS was found to be the best in increasing the solubility of P in 2% citric acid. Indications were also obtained that this substance could replace H$_2$SO$_4$ in the production of SP, and was believed to yield a more eco-friendly P fertilizer than conventional superphosphate.

ACKNOWLEDGMENTS

We greatly appreciate the financial support provided by the PT Petrokimia Gresik (Persero) under Research Collaboration Contract No. 105/03/01.02/46/SP/1997.
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