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

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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 phosphate-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 Morrocan 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 H_2SO_4 in superphosphate (SP) production and using both with lower H_3PO_4 concentrations were investigated with MPR as raw materials. Replacement of H_2SO_4 by the LCS in the SP production process yielded a comparable 2% citric acid–soluble P content. Combining the LCS and H_2SO_4 reduced the consumption of H_3PO_4 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 chemico-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, $Ca_3(PO_4)_2$ 5 g, NaCl 0.2 g, KCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $MnSO_4 \cdot 7H_2O$, $FeSO4 \cdot 7H_2O$ 0.0025 g, $(NH_4)SO_4$ 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

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

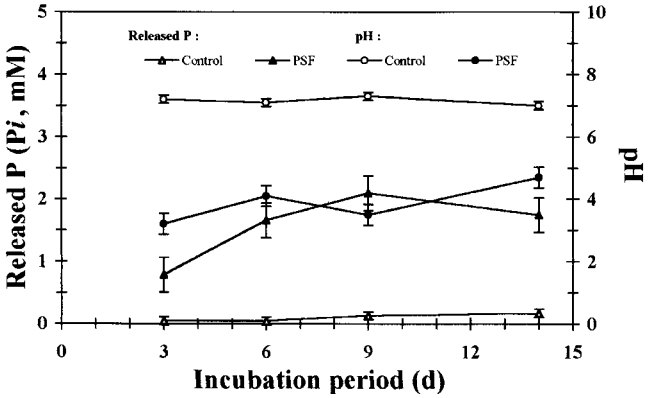

Fig. 1. The amounts of P released (P*i*) into the filtrate of Pikovskaya broth and pH of the medium during 14 d of phosphate-solubilizing fungus (PSF) culturing.

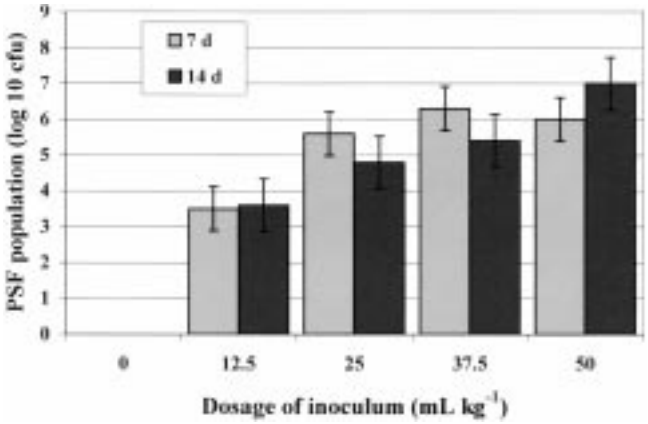

Fig. 3. Means of countable colony forming unit (log 10 cfu) on the Morrocan phosphate rock inoculated with various dosages of phosphate-solubilizing fungus (PSF) biomass at 7 and 14 d incubation.

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 commonly used 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 spectrophotometri-

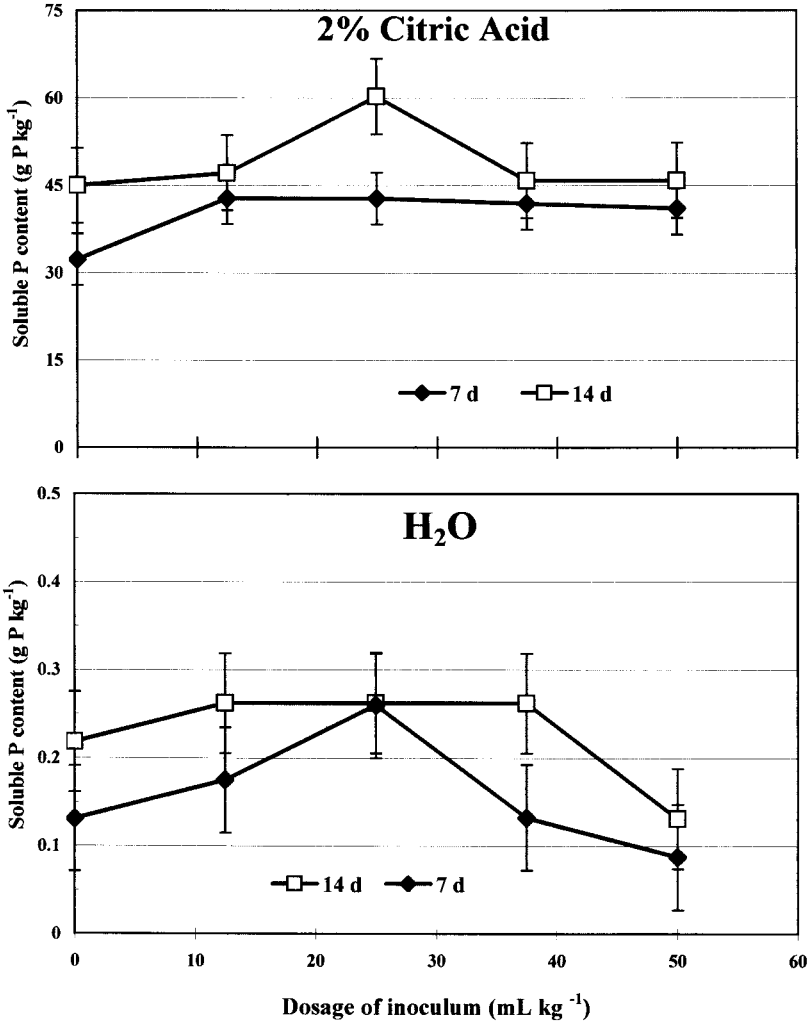

Fig. 2. Release of P from Morrocan phosphate rock treated with phosphate-solubilizing fungus biomass at varying dosages after 7 and 14 d of incubation, extracted with 2% citric acid (top) and water (bottom).

cally at 750-nm wavelength. Using standard P solutions from 0 to 1100 mg P kg^{-1}, we compared the Taussky and Shorr (1953) method to the molybdenum blue method of Olsen and Sommers (1982) and found that there was a good agreement in the P*i* values, as indicated by a regression R^2 value of 0.9999 and a slope of 1.01. The optimum incubation period was determined on the basis of soluble-P concentration data, and served as a reference in further experiments. Measurement of pH of the extract was conducted at the end of respective incubation period. To determine the optimum concentration of MPR as a P source in the PR solubilization, a similar experiment was conducted with 2.5, 5.0, and 10.0 g L^{-1} MPR in the modified Pikovskaya broth and shaken on a mechanical shaker at 100 rpm for 9 d at 28°C. The optimum concentration was used as a standard in the next experiment.

Bioactivation of Morrocan Phosphate Rock by Direct Inoculation of the Phosphate-Solubilizing Fungus on Morrocan 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 $10^{4.8}$ 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 Morrocan 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_2O_5 contents were determined by the methods previously mentioned. Similar experiments were conducted using H_2SO_4 and various concentrations of H_3PO_4, and by replacing H_2SO_4 with various concentrations of LCS. These experiments, simulating SP production by acidulation of MPR with H_2SO_4 and/or followed by H_3PO_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_2SO_4 (98%) and 3.25 mL H_3PO_4 adjusted at various concentrations, that is, 50, 100, 150, and 200 mL H_3PO_4 L^{-1}, by diluting H_3PO_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_3PO_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_3PO_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_2SO_4 (98%) and 280 mL H_3PO_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-

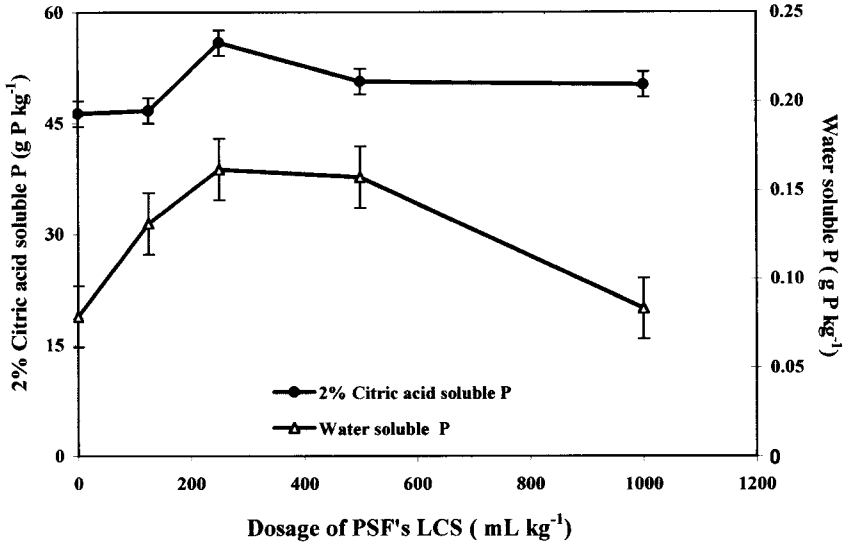

Fig. 4. Improvement on P solubility of the Morrocan phosphate rock treated with liquid culture supernatant (LCS) for 2 h at varying concentration. PSF is phosphate-solubilizing fungus.

Table 1. Soluble P contents of phosphate-solubilizing fungus's liquid culture supernatant (LCS) pretreated Morrocan phosphate rock at various concentrations of H_3PO_4 and addition of H_2SO_4 (98%).

	H_3PO_4 concentrations (mL L^{-1})							
	50		100		150		200	
LCS concentration	2% Citric acid	H_2O	2% Citric acid	H_2O	2% Citric acid	H_2O	2% Citric acid	H_2O
mL kg^{-1}	g P kg^{-1}							
0	74.4c p*	71.8c s	108.1a q	104.6a t	108.9c q	106.1ab t	123.6b r	116.3d u
125	95.7b p	89.8a s	111.9a q	100.6a t	113.9ab q	103.9bc t	126.0ab r	118.2c u
250	102.9ab p	89.4a s	112.9a pq	98.9a t	119.3a q	109.3a u	125.5a q	120.2a v
500	97.7ab p	83.6b s	109.1a pq	100.9a t	120.4a q	103.9bc t	126.9a q	119.1b u
1000	108.3a p	91.7a s	107.4a p	104.9a t	107.7bc p	126.7c t	126.7a p	115.1e u

* 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 ($P < 0.05$).

ferences of the mean values between treatments. Regression and correlation analyses were performed to determine the relationship between soluble P contents and fungal population and LCS concentrations and/or to determine the optimum level of the treatments studied.

RESULTS AND DISCUSSION

Effect of Incubation Period and Morrocan Phosphate Rock Concentration on Phosphate Rock Solubilization

The amounts of P released (P*i*) 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 P*i* were insignificant among the treatments of 2.5, 5.0, 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 μM citrate mL^{-1} medium, 1.6 μM malate mL^{-1} medium, 0.3 μM ptalate mL^{-1} medium, and 0.1 μM piruvate 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 μM 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 cytoplasma 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 P*i* 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 P solubility of treated MPR extracted by H_2O, 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 ($r^2 = 0.34^{ns}$). 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

Table 2. Soluble P contents of 100- and 200-mesh Morrocan phosphate rocks (MPRs) pretreated with 280 mL kg^{-1} H_3PO_4 (52%) affected by different type of activating agents.

Treatments	Soluble P content		Perchloric acid–extractable P (Rund, 1984)
	H_2O	2% Citric acid	
	g P kg^{-1}		
100-mesh MPR			
Untreated	0.08g†	50.5f	147.0c
170 mL H_2SO_4 (98%) kg^{-1}	121.2a	129.7bc	136.6de
85 mL LCS‡ kg^{-1}	81.0d	137.8b	167.7a
170 mL LCS kg^{-1}	102.6b	147.9a	169.9a
255 mL LCS kg^{-1}	52.6f	103.2d	164.4ab
200-mesh MPR			
Untreated	1.0g	65.8e	145.4cd
170 mL H_2SO_4 (98%) kg^{-1}	127.7a	131.0bc	134.1e
85 mL LCS kg^{-1}	93.2c	132.2bc	157.9b
170 mL LCS kg^{-1}	83.7b	137.6b	168.2a
255 mL LCS kg^{-1}	72.8e	126.6c	156.7b

† Figures followed by similar letter(s) in the same column are not significantly different according to DMRT ($P < 0.05$).
‡ 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 Morrocan Phosphate Rock

The standard operational procedure in SP production includes reaction of 200-mesh MPR with H_2SO_4 (98%) and H_3PO_4 (52%) (Young et al., 1985). The mass composition of these materials were 52% MPR and 48% acids (15.5% H_2SO_4 + 32.5% H_3PO_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_2SO_4 and H_3PO_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_2SO_4 (98%) and H_3PO_4 at selected concentrations. Another purpose of this experiment was to determine whether the use of the LCS in combination with H_2SO_4 may to some extent save the H_3PO_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_2SO_4, to some extent, but also to enrichment of P from H_3PO_4. Reacting a 500-mL LCS kg^{-1} pretreated MPR with H_2SO_4 (98%) appears to reduce the required concentration of H_3PO_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_2SO_4 (98%) in combination with H_3PO_4 (52%), both in 100- and 200-mesh MPRs (Table 2). Although the water-soluble P contents of MPR treated with LCS–H_3PO_4 were significantly lower than that treated with H_2SO_4–H_3PO_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_3PO_4 (52%). The observed data of soluble P contents at different LCS volumes ($n = 4$, excluding H_2SO_4 treatment) were highly fit to the quadratic equation model developed with $R^2_{H2O} = 0.99^{**}$ and $R^2_{\text{citric acid}} = 0.99^{**}$ for 100-mesh and $R^2_{H2O} = 0.97^{**}$ and $R^2_{\text{citric acid}} = 0.91^{**}$ for 200-mesh H_3PO_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_2SO_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_2SO_4 in the production of SP, and was believed to yield a more eco-friendly P fertilizer than conventional superphosphate.

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