Solubility Phosphorus Improvement of Indigenous Phosphate Rocks by Aspergillus Niger

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ABSTRACT

The effectiveness of phosphate-solubilizing fungi (PSF) in improving phosphorus (P) solubility of poorly-soluble phosphate rock (PR) is assumed to be dependent on the suitability of the fungal isolate to the mineralogical suite of the rocks. A laboratory study was carried out to bioactive two mineralogically different hardly-soluble apatitic indigenous PRs by using an organic-acid-producing *Aspergillus niger* BCC F.194. Optimum fungal culturing conditions for P solubilization were determined by varying the carbon and nitrogen contents of the Pikovskaya broth medium at different shaking speeds. Liquid cultural supernatant (LCS), obtained from the optimum conditions was then reacted with 55g PR, i.e. Cileungsi and Madura PRs, for the two hours at various concentrations, i.e. 8.5, 17, 25.5, and 34 ml; in combination with 28mL H_3PO_4 (52%) addition. Without LCS addition and conventional superphosphate treatments acted as negative and positive checks, respectively. Phosphate determination included water, 2% citric acid, and perchlorate-soluble P contents. Mineralogical analyses were performed by x-ray diffractometer (XRD), differential thermal analyzer (DTA), and scanning electron microscope (SEM). The use of oxalic-acid-containing LCS in combination with H_3PO_4 increased significantly P solubility of both PRs due to the destruction of hydroxy-and chlorapatite crystals caused by acidification. Hydroxyapatite exhibited a higher response to the treatment than the chlorapatite did. As the soluble P contents are comparable to that of conventional superphosphate, the production of the P fertilizer with a lower acid consumption is possible. To overcome low reactivity of natural phosphate rocks (PR) as P source, several alternatives have been proposed. These include partial acidulation (Hammond *et al.*, 1986; Bolan *et al.*, 1997; Lewis *et al.*, 1997; Rajan *et al.*, 1997), synthetic organic acid activation (Sagoe *et al.*, 1998), natural organic acid reaction (Singh and Amberger, 1998 a&b), and decreasing particle size (Goenadi, 1994; Babare *et al.*, 1997). All of these approach were intended to increase P availability to crops grown on acid soils. However, increasing pressure on not-using synthetic chemical products within the last two decades retards the wide application of such alternative. Moreover, endless rise on production cost of conventional superphosphate (SP) echos the need of a more environmentally safe and economically feasible technology.

More efforts were then spent to utilize microorganisms, especially bacteria and fungi, for improving dissolution of PR. Thien and Myers (1992) showed that bioavailibility of P in a bioactive soils was remarkably enhanced by increasing soil microbial activities. Others have reported that certain soil microbes are capable of solubilizing relatively insoluble phosphatic compounds (Asea *et al.*, 1988; Nahas *et al.*, 1990; Goenadi *et al.*, 1995; Bojinova *et al.*, 1997). These phenomena have been attributed to the fact that increasing P solubilization of PR was closely related to the ability of the microbes in producing selected organic acids (Kucey, 1983; Illmer and Schinner, 1992; Goenadi *et al.*, 1993; Omar, 1998; Kim *et al.*, 1998). Citrate and oxalate were common organic acids reported in these literatures as well as that reported earlier by Banik and Dey (1982). However, the types of organic acids produced have been reported to be dependent on the chemical suites of the culturing medium (Cunningham and Kuiack, 1992), and consequently affecting the effectivness in P solubilization.

Recent study conducted by Goenadi et al., (2000) indicated that a P solubilizing fungus (PSF) *Aspergillus niger* grown on Pikovskaya broth enriched with Morrocan PR (MPR) produced citrate, malate, ptalate, and piruvat. By applying the liquid culture supernatant (LCS) on MPR, they showed that SP may be produced without sulfuric acids addition. However, the effect of this so-called bioactivation technique on mineralogical change of the PR resulting in the increase of soluble P content has not been clarified. Besides, the consistency of such technique on different types of PR need to be determined. Our objectives was to bioactive two mineralogically different hardly soluble PRs by using an organic-acid-producing *A.niger* in attempt to develop an effective technique for the production of environmentally safe P fertilizer.

INTRODUCTION

Indonesia considered as agricultural country still needs many kinds of fertilizer in huge number. Among the conventional fertilizer, the need of phosphate fertilizer (P) in 2003 almost 2.83 million tons with the value of 2.83 billion rupiahs ($35.4 M). Unfortunately, almost all of the component is imported materials, especially the natural phosphate as a raw material. Eventhough the domestic natural phosphate is almost one million ton, however its quality is poor due to non reactivity and inconsistent.

The effort to increase the reactivity has been done by utilizing sulfuric acid or phosphoric acid. This technology is expensive and also environmental unfriendly. An applied microbiology research results has provided the opportunity to utilize the microorganism as an activator in P solubilizing from natural phosphate. This microbe can be able to produce the phosphatase enzyme and organic acid for P solubilizing.

Based on the initial research, it is known that citric acid is the dominant agent for P-FAM and FAC in liquid culture supernatant (LCS) from Aspergillus niger BCC F.194. By using this techniques, it is expected that the supply of P for can be increased without increasing the doses of P fertilizer. The utilizing of direct microbe is not efficient due to the needs of time for incubation (Goenadi, 1998). Therefore, the target of this research is the package of technology utilizing the A. niger for solubility improvement in activation of local natural phosphate.

The objectives of the study are (1) to optimize fungal culturing conditions, (2) to observe the effect of organic acids on PR dissolution and its bioactivation.

MATERIALS AND METHODS

The step of research were divided into several steps, i.e. (1) optimizing fungal culturing conditions for LCS production, (2) effect of organic acids on PR dissolution, (3) bioactivation of PR by LCS addition and (4) mineralogical analyses.

Optimizing fungal culturing conditions for LCS production

Optimum conditions for A. niger BCC F.194 (Goenadi et al., 2000) cultivation were achieved by varying the C and N contents of the PR-containing Pikovskaya broth medium combined each with different mechanical shaking speeds, i.e. 40, 70 and 100 rpm. Glucose was used as C source given at three levels, i.e. 0.5, 1.0, and 1.5% (w/v), whereas $(NH_4)_2SO_4$ served as N source, i.e. 0.025, 0.05 and 0.1% (w/v). A series of 250 mL Erlenmeyer flasks containing 100 mL of Pikovskaya broth enriched separately with 0.25% (w/v) Cileungsi PR (CPR) and Madura PR (MPR) were inoculated by a loop of a 7-day-old agar (Oxoid L11) plate culture of the fungus and incubated for 9 days at room conditions. Chemicals data of MPR were 71.2 $g.kg^{-1}$ perchlorate-soluble P, 39.1 $g.kg^{-1}$ citric acid-soluble P, 0.1 $g.kg^{-1}$. water-soluble P, 253.8 $g.kg^{-1}$ CaO, and 161.1 $g.kg^{-1}$ chlorine (Cl), whereas those of CPR were 88.8$g.kg^{-1}$, 48.6$g.kg^{-1}$, 0.1$g.kg^{-1}$, 115.5$g.kg^{-1}$, and 41.4 , respectively. Subsequently, the optimum level of C source was applied to determine the optimum level of N source at optimum level of aeration obtained by mechanical shaking speed, and finally these two optimum level of nutrient sources were applied to determine the optimum PR concentration for optimum growth of P solubilizing ability, and organic acid produced by fungus. Five level or respective PR concentrations were 0, 1.25, 2.5, 5.0 and 10.0 $g.L^{-1}$. The growth of the fungus was measured by mycelia dry weight at the end of incubation period. Phosphorus solubilizing ability was determined by measuring the soluble-P by molybdenum blue method (Olsen and Sommers, 1982) and expressed it as percentage to perchlorate-soluble P of the rock. Organic acids was quantified by high performance liquid chromatography (HPLC) technique.

Effect of Organic Acids on PR Dissolution

This experiment was carried out to clarify the relative strength of different types of organic acids in solubilizing P from the PRs used. Citrate, oxalate, and gluconate were separately reacted with CPR and MPR at six levels, 0, 0.05, 0.50, 1.0, 3.0 and 6.0 mM. Each organic acid was added to 50 mL autoclaved water containing 1.25 $g.L^{-1}$ sterilized CPR or MPR. The mixture was then mechanically shaken at 100 rpm for seven days under room temperature (Illmer *et al.*, 1995). Perchlorate-soluble P was determined at the end of incubation (Rund, 1984).

Bioactivation of PR by LCS Addition

The LCS obtained from a nine-day old culture of *A. niger* under the most optimum cultivation conditions resulted from previous experiments was then used to activate CPR and MPR. Following the procedures reported earlier (Goenadi, *et al.*, 2000), 55 g of non-sterilized respective PR were reacted with 0, 8.5, 17, 25.5 and 34 mL LCS in 250 mL erlenmeyer flasks for 2 h. on a mechanical shaker at 100 rpm. The liquid solid ration was kept at 1:1 (v/w) by making up the volume with sterile deionized water by using the methods previously mentioned (Olsen and Sommers, 1986). The most optimum LCS concentration was selected on the basis of these parameters. Subsequent experiment was carried out to produce superphosphate by using the LCS-activated PRs. A 5.2 g of 80-mesh LCS-pretreated CPR and MPR was reacted with 28 mL H_3PO_4 (52%) (Young *et al.*, 1985) for 2 h. and dried by using oven drier. These experiments were arranged in complete Random Design with two replicates. Perchlorate, 2%-citric acid-, and water-soluble P were observed by using the methods mentioned previously.

Mineralogical Analyses

Mineralogical analyses were conducted by using x-ray diffrectometer (XRD, Shimadzu), differential thermal analyzer (DTA, SETARAM), and scanning electron microscope (SEM, PHILLIPS 515). A 200-mesh-size powder was prepared prior to the analyses. The samples included those of LCS treated, or untreated, and of conventional SP (SP-36, PT Petrokimia Gresik) which were constructed from both of CPR and MPR. Parallel-oriented sample were performed for XRD analysis using Cu-Kα radiation at 30 kV and 30 mA as reported by Brindley and Brown (1980) and Goenadi (1991). DTA was performed with one mg sample against two mg inert Al_2O_3 employing a heating rate 15°C/min (Goenadi. 1989). Micro visualization of sample was carried out by SEM observation after gold coating by TAAB coating sputter (Goenadi and Tan, 1991).

Statistical Analyses

Statistical analyses was conducted using Duncan Multiple Range Test (DMRT) (P=0.05) to determine significant difference of the mean values between treatments. Regression and correlation analyses were performed to determine the relationship between soluble P contents and pH of the medium, fungal, biomass, and organic acid concentration.

RESULTS AND DISCUSSION

Optimizing Fungal Culturing Conditions for LCS Production

Optimum condition for fungal growth in in-vitro medium are influenced by the composition of the medium, particularly C and N concentrations (Narisan *et al.*, 1995). The rate of oxygen supply into the culturing medium was also reported affecting the growth of fungal isolate, such as *Aspergillus niger* (Illmer and Schinner, 1992). Our data showed in agreement with these phenomenon. The highest ability of A. niger BCC F.194 isolate used in solubilizing P of CPR and MPR was achieved at 100 $g.L^{-1}$ glucose, 50 $g.L^{-1}$ $(NH_4)_2SO_4$, and shaking speed of 100 rpm (MPR) and 70 rpm (CPR) (Fig 1).

Phosphate solubilization mediated by phosphatase enzyme is believed to be taken for organic P sources (Spiers and McGill, 1979; Bishop *et al.*, 1994), these investigators and others considered that the inorganic P biosolubilization is governed by the organic acids produced by P-solubilizers. Employing the optimum C and N concentration of Pikovskaya medium, the A. niger BCC F.194 produced oxalic acid as the main organic acids ranging from 0.06 to 3.75 mM. These values, obtained by using HPLC (0.01 N H_2SO_4 mobile phase, 210 nm UV detector, 0.5 $mL.min^{-1}$ flow rate, at 50°C was resulted from MPR or CPR as P-source of Pikovskaya medium at 12.5 $g.L^{-1}$ level. Figure 2. shows the relationship among PRs concentration and P-solubilizing ability and either pH of the medium, fungal biomass, or oxalic acid produced. Regression analysis indicates that P-solubilization was highly correlated with oxalic acid concentration ($r_{MPR} = 0.92^{**}$ and $r_{CPR}=0.85^{*}$) as well as with the pH of the medium ($r_{MPR} = -0.87^{*}$ and $r_{CPR}=-0.99^{**}$). Our data also showed that oxalic acid concentration were strongly correlated with the pH of the medium ($r_{MPR} = -0.99^{**}$ and $r_{CPR}=-0.87^{*}$).

Effect of Organic Acids on PR Dissolution

To confirm the relationship between organic acid concentration and the P-PRs solubilization, a range of synthetics organic acids concentration (up to 6 mM), i.e. oxalic, citric, and gluconic acids, was reacted with MPR and CPR. It was evident that oxalic and citric acids have strong influences to the solubilization of P-PRs, i.e. $r_{MPR} = 0.96^{**}$ and $r_{CPR}=0.97^{**}$(oxalic acid) and $r_{MPR} = 0.96^{**}$ and $r_{CPR}=0.99^{*}$ (citric acid). These phenomenon lead to the assumption that oxalic acid produced by *A.niger* BCC F.194 isolate is responsible for lowering the pH of medium providing more protons (H^+) to increase the P-PR solubilization. This assumption is based on what Illmer and Schinner (1992 and 1995) have postulated that the production of organic acids is an important mechanisms for solubilizing relatively insoluble P, but they believed that this is not the only possible one. Another 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 H^+ translocation ATPase which is located in the plasma lemma and uses the energy for ATP hydrolysis.

Following the above hypothesis, those 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. By using the same fungal isolate, Goenadi *et al.*, (2000) showed that the amount of soluble P of Morrocan phosphate rock was closely related to the fungal biomass within 14 days of culturing. Our current findings indicate interesting evidence. As indicated on Fig 2. the highest amount of soluble P-MPR was achieved at pH 4 to 5, whereas the value was obtained at pH=2 for P-CPR. This differences implies the pH buffering capacity of the rocks. Cileungsi PR has lower pH buffering capacity that of Madura PR as the later contained calcium (18.3% Ca^{++}) higher that the former (8.3% Ca^{++}).

Bioactivation of PR by LCS Addition

Data shown in Table 1 provide evidence in which the addition of LCS improved the water-soluble P of l PRs investigated. In contrast, improvement on citric acid-soluble P was only obtained from LCS-pretreated C Practically, the improved water-soluble P is not significant as it was much less than 10 g.kg^{-1}. However, this incr indicate to some extent the degradation of P-bearing mineral due to the addition of oxalic-acid-containing LCS. both PRs the optimum LCS treatments was achieved at 170 mL LCS.kg^{-1} addition. This level was then used for fur experiment.

In commercial practice superphosphate (SP) is produced by reacting a 200-mesh PR (P_2O_5>280g.kg^{-1}) H_2SO_4 (98%) and H_3PO_4 (52%) (Young *et al.*, 1985). The mass composition of these materials are 52% PR and acids, i.e. 15.5% H_2SO_4 and 32.5% H_3PO_4. However, some variations in the mass composition may occur at indus practices depending on the grade of the rock and/or the acids. Goenadi *et al.*, (2000) has shown that the acidulation H_2SO_4 can be potentially replaced by LCS in the formulation of Morrocan-PR-originated SP. Data presented in Tab show that the addition of LCS replacing H_2SO_4 input significantly improved the P solubility of both MPR and C Although this improvement in water-soluble P was still lower than those yielded by the conventional technique, but increase in citric- acid-soluble P was either similar or higher. These results were in agreement with those repo previously (Goenadi *et al.*, 2000). Considering the higher contents of perchloric-acid-extractable P of the I pretreated PRs compared to those of standard process, the bioactivation of PR may be conducted by lowering concentration of H_3PO_4.

The improvement of P-solubility of acidulated or bioactiv
bioactivated PR is assumed to be related with the degradation of the P-bearing minerals, i.e. hydroxyapatite chlorapatite, due to acid introduction. Stoichiometrically, acidulation introduces an acid into the system which attack the stability of apatitic minerals. Introduction of H^+ ion is capable of altering the crystallographic structure of minerals, possibly shortening the mineral's axes and/or decreasing crystalline size, resulting in a more P_2O_5 in solt form (Goenadi *et al.*, 2000). Mineralogical analyses indicate that acidulation caused the reduction of XRD p intensity of apatitic minerals (Fig 3.). On the other hand, the treatment intensified the two main endothermic pe present at 150-200 °C and 250-400°C temperature (Fig. 4). These phenomena may be attributed to the reductio crystallite size as evidenced by the SEM microphotographs (Fig. 5). As shown in Fig. 5, these acidulation either by L + H_3PO_4 or H_2SO_4 + H_3PO_4 resulted in the formation of smaller crystallite of P-bearing minerals.

CONCLUSIONS

The utilization of oxalic-acid-containing LCS, produced by nine-days-old culture of A. niger BCC F.194 combination with H_3PO_4 increased significantly soluble P contents of both MPR and CPR. Improved solubility of th indigenous PRs was attributes to the destruction of hydroxy-and chlorapatite crystals caused by acidulation. Employ this LCS bioactivation technique, a more ecological friendly P fertilizer than conventional SP can be yielded. Howe the agronomic effectiveness of this product requires further studies.

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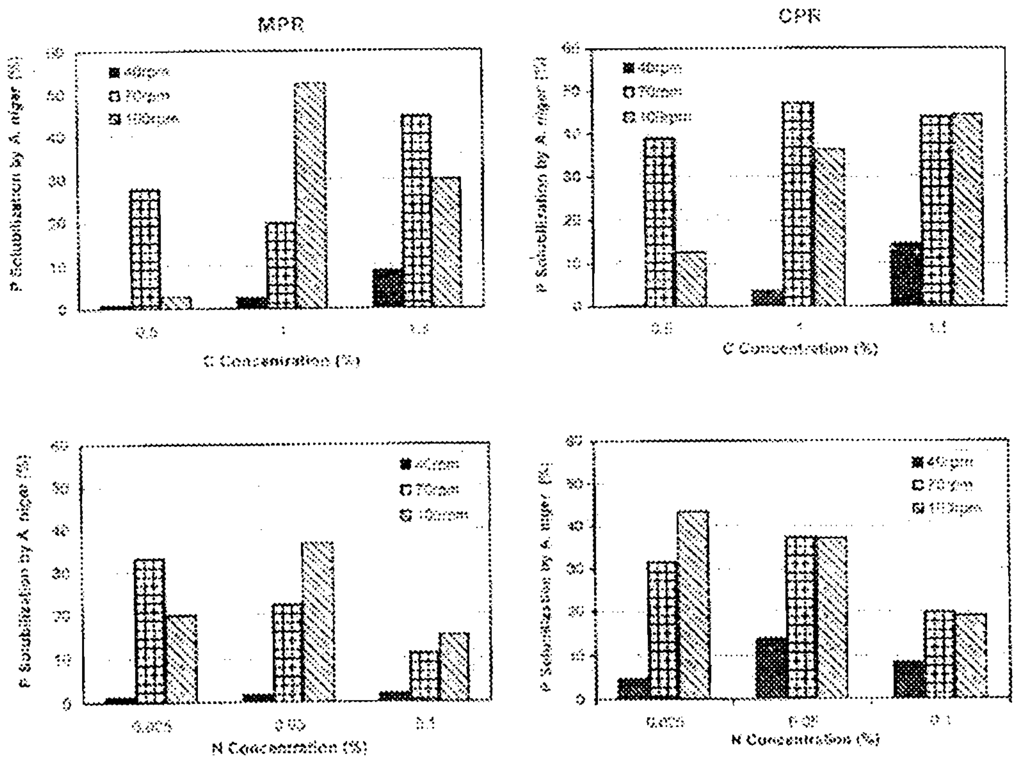


Fig. 1. MPR- (left) and CPR-(right) solubilizing ability of *A. niger* BCC F.194 at different shaking rate (rpm), carbon concentration (above), and nitrogen concentration (below)

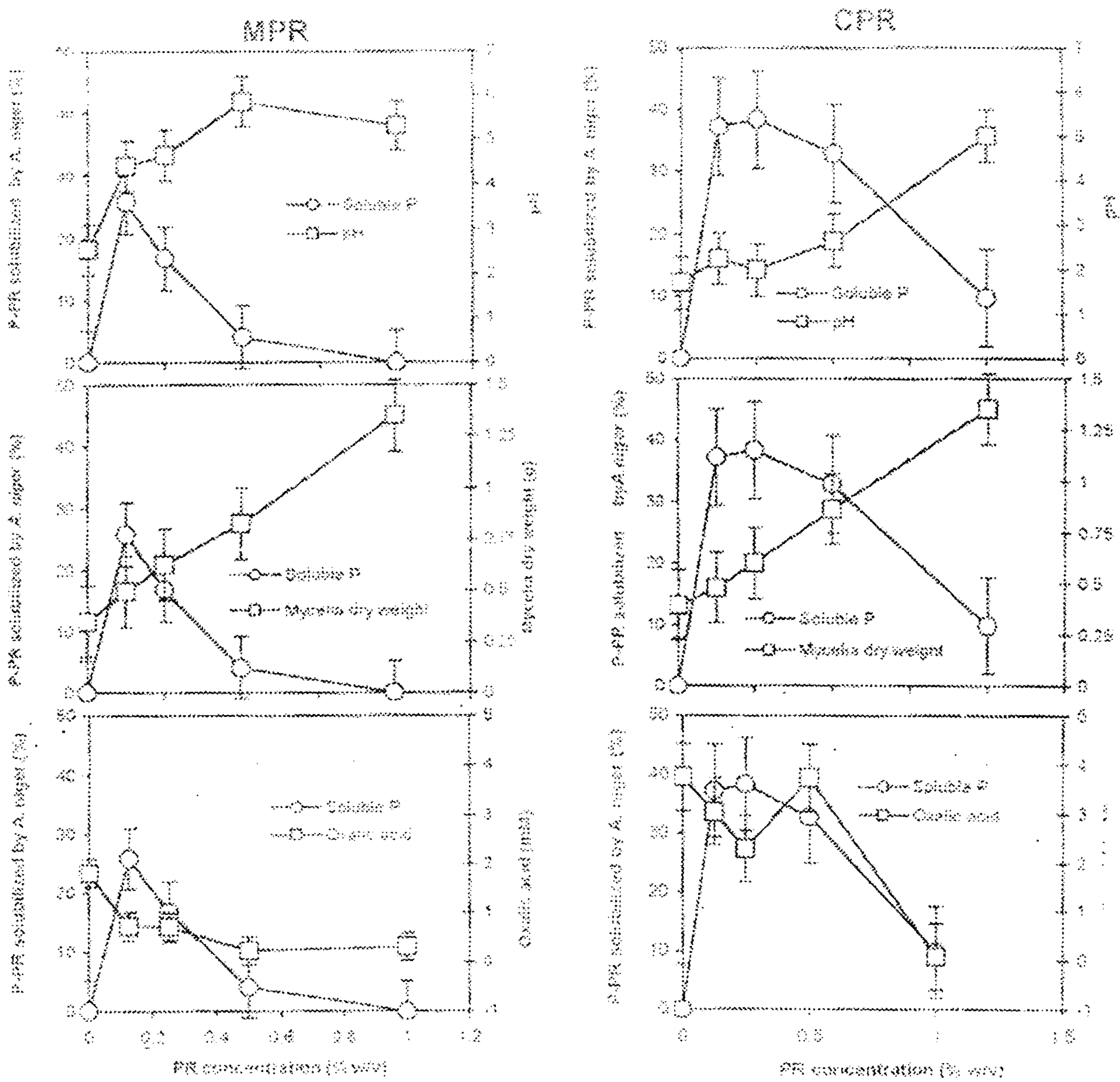


Fig. 2. The dynamics of P-PR solubilization and pH (top), fungal biomass (middle), and oxalic acid produced (bottom) at different levels of MPR (left) and CPR (right) concentrations after 9 d of culturing.

Fig. 3 . X-ray diffractograms of MPR and CPR samples: untreated PRs, LCS-pretreated PRs , LCS + H3PO4 treated PRs, and chemically acidulated PRs.

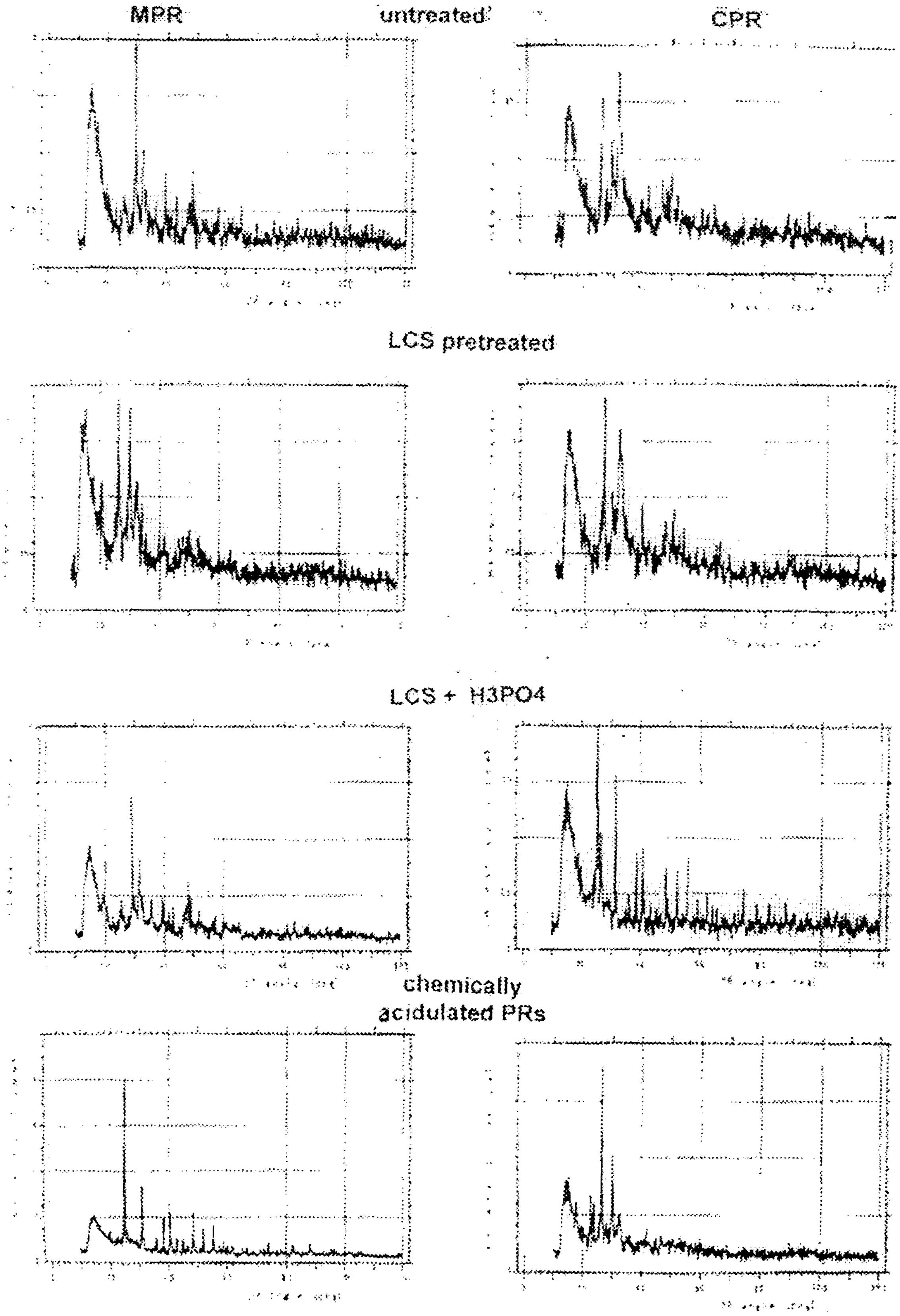
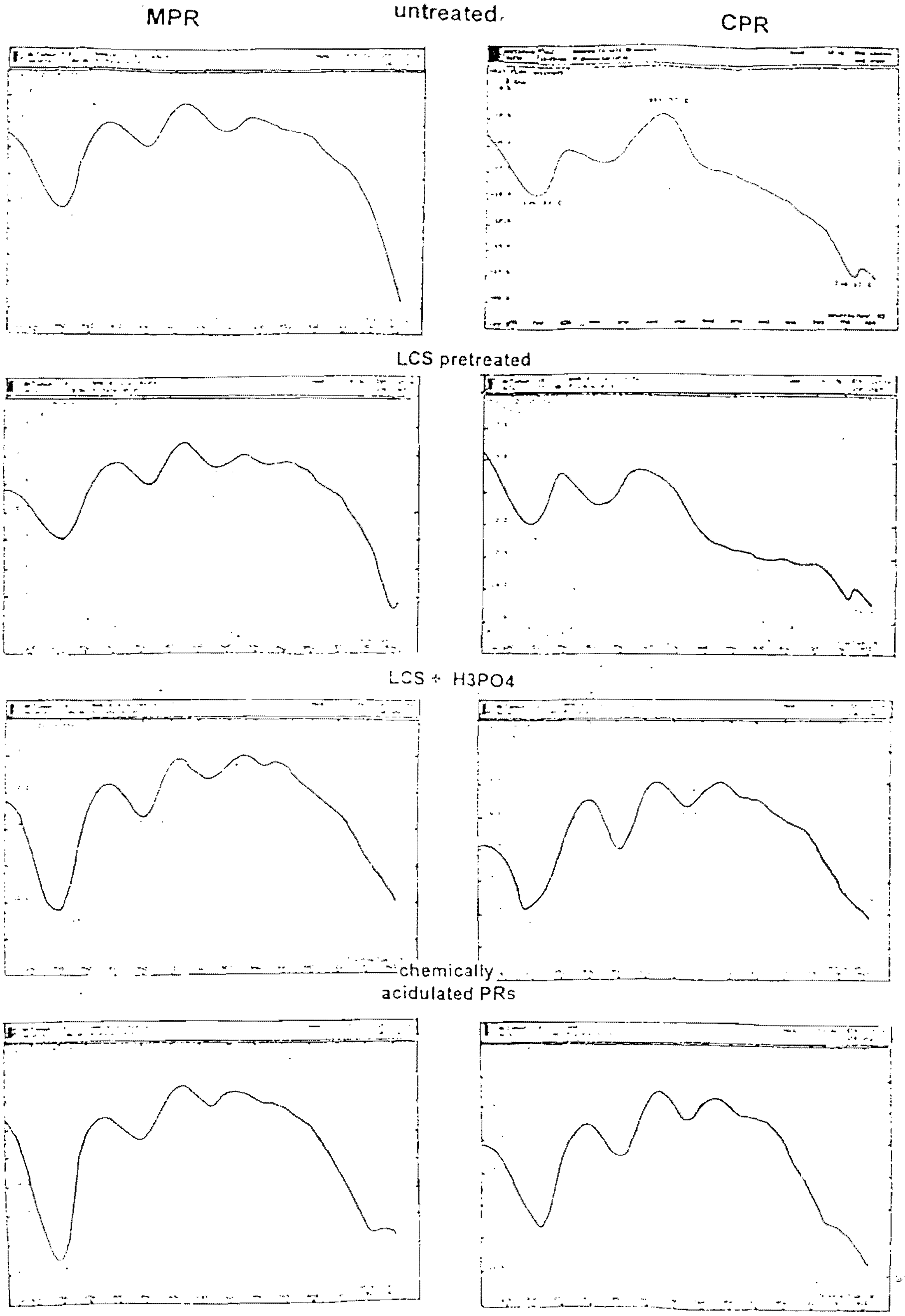


Fig. 4 . DTA curves of MPR and CPR samples: untreated PRs, LCS-pretreated PRs , LCS + H3PO4 treated PRs, and chemically acidulated PRs.



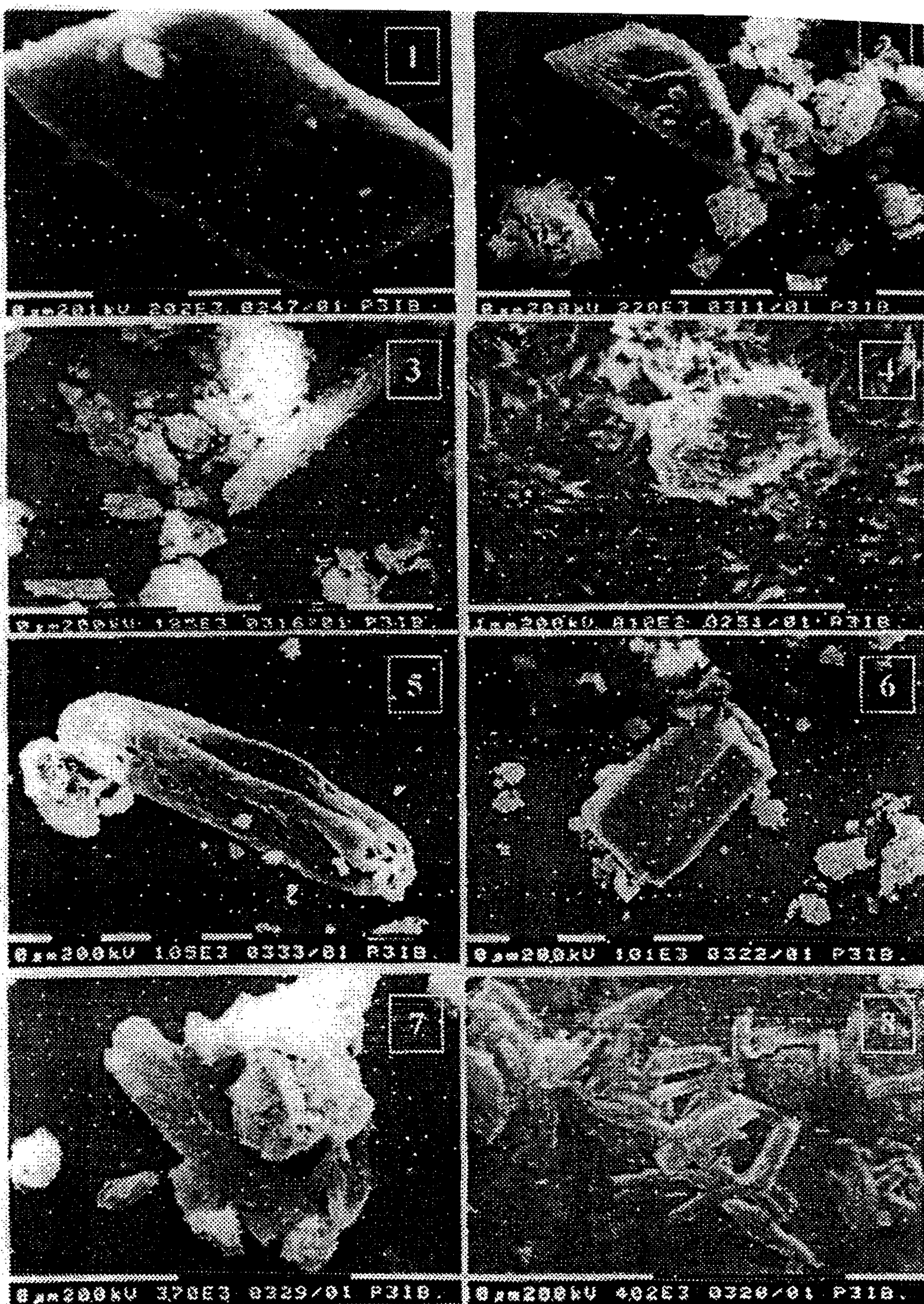


Fig. 5 . Scanning electron microphotographs of MPR and CPR samples: untreated PRs (1 & 5), LCS-pretreated PRs (2&6), LCS + H3PO4 treated PRs (3&7), and chemically acidulated PRs (4&8).

Tabel 1. Average of P_2O_5 (perchlorate-soluble, water-soluble, and citric acid-soluble) MPR and CPR activated by using LCS.

Treatments	P_2O_5 Extr. (g.kg^{-1})		
	perchlorate	water	citric acid
MPR	135 a	0.19 c	99 a
MPR+85 mL LCS	144 a	0.19 c	105 a
MPR+170 mL LCS	141 a	0.24 b	105 a
MPR+255 mL LCS	138 a	0.21 c	104 a
MPR+340 mL LCS	134 a	0.29 a	99 a
MPR+1000 mL LCS	131 a	0.20 c	101 a
CPR	200 a	0.1 b	111 b
CPR+85 mL LCS	202 a	0.18 b	158 a
CPR+170 mL LCS	191 a	0.43 a	166 a
CPR+255 mL LCS	199 a	0.13 b	166 a
CPR+340 mL LCS	192 a	0.22 b	165 a
CPR+1000 mL LCS	204 a	0.15 b	163 a

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

Tabel 2. Average of P_2O_5 (perchlorate-soluble, water-soluble, and citric acid-soluble) MPR and CPR activated by using $LCS+H_3PO_4$ or $H_2SO_4+H_3PO_4$

Treatments	P_2O_5 Extr. (g.kg^{-1})		
	perchlorate	water	citric acid
MPR	163 c	0.3 d	89.5 d
MPR+85 mL $LCS+H_3PO_4$	346 a	128 b	326 ab
MPR+170 mL $LCS+H_3PO_4$	329 a	121 b	301 ab
MPR+255 mL $LCS+H_3PO_4$	309 ab	86 c	285 bc
MPR+340 mL $LCS+H_3PO_4$	353 a	163 a	345 a
$MPR+H_2SO_4+H_3PO_4$	270 b	171 a	242 c
CPR	203 e	0.2 e	111 c
CPR+85 mL $LCS+H_3PO_4$	379 ab	199 b	334 a
CPR+170 mL $LCS+H_3PO_4$	359 bc	189 b	326 a
CPR +255 mL $LCS+H_3PO_4$	336 cd	161 c	313 a
CPR+340 mL $LCS+H_3PO_4$	398 a	127 d	315 a
$CPR+H_2SO_4+H_3PO_4$	311 d	267 a	272 b

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