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Process of rock phosphate solubilization by *Aspergillus* sp PS 104 in soil amended medium

S.C. Kang[1], Piyush Pandey[2], Rajat Khillon[3] and D.K. Maheshwari*[3]

[1]Division of Food, Biological and Chemical Engineering, College of Engineering, Daegu University, Gyeongsan City - 712-714, Republic of Korea
[2]Department of Microbiology, SBSPGI of Biomedical Sciences and Research, Balawala, Dehradun - 248 161, India
[3]Department of Botany and Microbiology, Gurukul Kangri University, Hardwar - 249 404, India

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Abstract: *Aspergillus sp PS 104, a soil isolate had excellent potential to solubilize rock phosphate in vitro. The process was influenced by the presence of various concentrations of local loess (red soil). The simultaneous occurrence, in our experiment, of high levels of solubilized phosphate and synthesized citric acid, together with the lowest reached pH values, confirmed the role of citric acid in the phosphate solubilization mechanism. When the soil was present, phosphate release was better correlated than citrate synthesis with H^+ concentration. Changes in soluble phosphate concentration did not follow a sigmoid pattern. The ability of organism to release phosphatase was also studied. An interesting relationship was observed between the two processes of phosphate mobilization: citric acid synthesis and phosphatase production.*

Key words: *Aspergillus sp PS-104, Phosphate solubilization, Loess, Citrate, Phosphatases*
PDF of full length paper is available with author ***(*maheshwaridk@gmail.com)***

Introduction

Conditions like irregular rainfall, long dry and hot summers and man-mediated degradative activities (overgrazing, non-regulated cultivation techniques, deforestation, *etc*) results in the disturbances of soil ecosystems (Misir *et al*., 2007). Such degraded ecosystems are usually characterized by a disturbed vegetation cover accompanied by a rapid erosion of surface soil (Herrera *et al*., 1993). The desertification process involves a loss or reduction of major physicochemical and biological soil properties (Requena *et al*., 2001), including its mineral content. In fact, despite being abundant in soil, both in inorganic and organic forms, phosphorus (P) is the major plant growth–limiting nutrient after nitrogen (N). Plant mineral nutrition depends mainly on the phosphorus content of soil, which can be assimilated only as soluble phosphate. Physical and chemical weathering of mineral phosphates is mainly realized along plant roots in the rhizosphere. Phosphorus in soils is present in insoluble form complexed with cations like iron, aluminum and calcium. Although, use of chemical fertilizers for improving soil fertility is the common approach of increasing agricultural production, a large portion of inorganic phosphates applied to soil as fertilizer is rapidly immobilized after application and becomes unavailable to plants (Yadav and Dadarwal, 1997). Microorganisms substantially influence the soil productivity by solubilizing this insoluble P through their metabolic processes in soil (Ravikumar *et al*., 2007; Sahu *et al.,* 2007). The process of microbe mediated P solubilization is generally ascribed to the production of organic acids by them (Fleischer *et al*., 1988; Das *et al*., 2003; Mattey, 1992; Tarafdar *et al*., 1988; Boavida and Heath, 1986; Illmer *et al*., 1992). Still there are reasonable doubts on the exclusive role of organic acids in solubilization process (Asea *et al*., 1988; Illmer and Schinner, 1995).

Use of rock phosphate (RP) as fertilizer for P-deficient soils has received significant interest in recent years since they are natural, inexpensive and available fertilizers. However its solubilization rarely occurs in non-acidic soils (Caravaca *et al*., 2004; Ouahmane *et al*., 2007). Physical and chemical treatment like thermal alteration and partial acidification, for increasing the P availability from RP are cost intensive. A much cheaper and convenient alternative is reclamation through use of P solubilizing microorganisms. In the light of these facts, the present study was undertaken to investigate the process of RP solubilizing activity of *Aspergillus* sp PS-104 and also, effect of local soil on this process. It is known that potentially useful organisms reported in the scientific literature fail in natural environment and never appear in commercial market (Bashan, 1998). Since soil influences the establishment and activity of the inoculated microbes, we included the local red soil (loess) with low P content in P solubilization experiments. The changes in pH and soluble P concentration along with citric acid production, occurring during solubilization of insoluble RP was determined and analyzed for the influence of loess during P solubilization process. Microbial activities in soil also result in mineralization of organic phosphates, which is the reverse process of solubilization. Mineralization results in reduction of soluble phosphate pool available to plants, by converting organic P in insoluble forms. As *Aspergillus* sp PS-104 was able to release phosphatase, its effect on RP solubilization process was also studied.

Materials and Methods

PS 104 was isolated from a farmer's field at Taegu (South Korea) along with several other fungal strains. Suitable dilutions of soil were plated on Potato dextrose agar (PDA) (pH 6.5), supplemented with 0.5% (w/w) calcium phosphate. A clear halo

zone around developing colonies in otherwise turbid medium was considered as positive for phosphate solubilization (Pikovskaya, 1948). Strain PS 104, was selected for further studies as it had maximum ability to solubilize inorganic phosphate indicated by the zone size diameter. The isolate was identified as *Aspergillus* sp by its cell morphology and ability to form spores (Gilman, 1956). The culture was maintained on slants as described (Pikovskaya, 1948).

50 ml Potato dextrose broth (PDB) of pH 6.5 was supplemented with 0.5% (w/w) RP. It was inoculated with 1 ml of spore suspension (approx. 1.2 x 10^6 spores ml^{-1}) in 250 ml Erlenmeyer flasks. The flasks were incubated at 28^0 C for 15 days. Samples were withdrawn periodically in aseptic conditions and centrifuged at 8000 rpm for 10 min. The clear solution was utilized for detection of pH, citrate, quantitative amount of soluble P (Olsen and Sommers, 1982) and phosphatase (Tabatabai and Bremner, 1969). One enzyme unit of phosphatase was defined as amount of enzyme that hydrolyzed 1mM of p-nitrophenol phosphate sec^{-1}. Un-inoculated flasks from each set were treated as control. Different concentrations of sterilized loess (0.1%, 0.5%, 1.0% w/v) were included in above-mentioned medium to observe the effect of local soil on the process. Physically soil was silt clay loam, gleysol over calcareous loess with acidic pH (5.9). The content analysis of soil resulted in 2.8 % calcium, 0.3 % potassium, 0.5 % sodium, 0.0 % magnesium and 0.3 % organic matter. The P content was also very low (0.01 %). The constituents of loess were constant irrespective to region (Jinryang, Kyungsan, Taegu) of Kyungbook area. Loess was not added in control flasks.

Citric acid production was estimated by high performance liquid chromatography (HPLC) analysis. HPLC operating parameters for the analysis of organic acids were: Instrument: Varion/ LS star; Column: Hypersil $BDSC_{18}$; Detector: UV 210 nm; Mobile phase: 0.1% H_3PO_4; Flow rate: 0.7 ml min^{-1}; Column temperature: 30°C and Injection volume: 20 µl.

The soil was analyzed by using standard methods (Han *et al*., 1988). All experiments were performed in triplicate and results reported as average. The t-scores were determined and contrasted to confidence level of 0.05, for statistical analysis.

Results and Discussion

Significant amount of insoluble RP was solubilized by the isolate as evident by soluble P concentration (Fig. 1) after different intervals of incubation. The trend of free P release was similar for all the concentrations of loess. Soluble P concentration increased till 168 hr, which decreased on 172 hr and remained almost constant thereafter. Maximum amount of P was released on 168 hr. Different concentrations of loess had considerable effect on the process. Almost linear relationship was observed in increase of maximum soluble P released with different concentration of loess (Fig. 1). Maximum 425 ppm of soluble P was released at 1% loess as compared to 266 ppm, released in absence of loess after 168 hr.

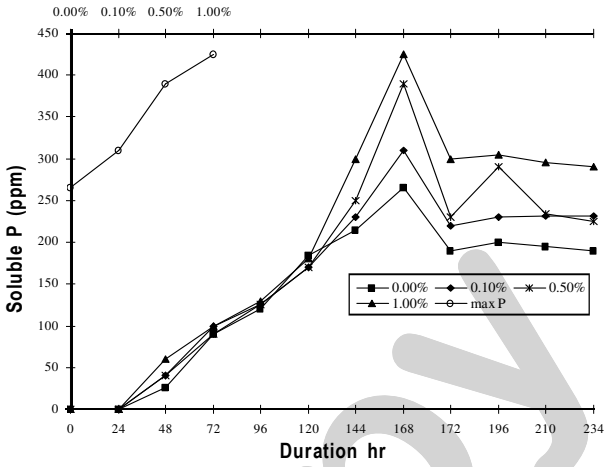

Fig. 1: Change in soluble P concentration during culture of *Aspergillus* sp PS-104 with various concentration of loess

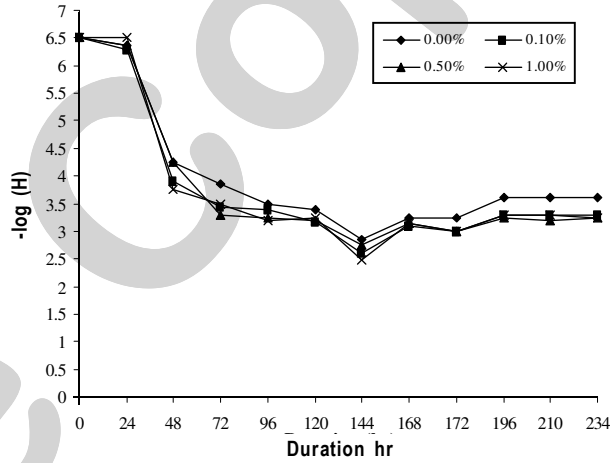

Fig. 2: Change in pH during culture of *Aspergillus* sp PS-104 with various concentration of loess

Aspergillus aculeatus (Narsian and Patel, 2000) and *Aspergillus niger* (Cerezine *et al*., 1988; Caravaca *et al*., 2004) have been studied earlier as RP and fluoroapatite solubilizers respectively, where decrease in pH was invariably found to be associated with solubilization process in both the cases, which is in accordance to our findings. However, no significant relationship was established between the quantities of phosphate solubilized and decrease in pH in the former case (Narsian and Patel, 2000). However, in present study more than 80% correlation was observed between them in absence of loess. Negative value refers that decrease in pH corresponds to respective increase in soluble P concentration. Earlier works have resulted in mixed information about the microbial solubilization of insoluble phosphates. Some authors have credited release of organic acid for the solubilization of P (Cunningham and Kuiack, 1992; Kucey, 1983), while others had reasonable doubts on the exclusive role of organic acids in solubilization mechanism (Asea *et al*., 1988). Even those authors, who believe in organic acid theory are not or hardly able to find a

Table - 1: Correlation coefficients between soluble phosphate - pH, citrate and phosphatase

Loess concentration (%)	Correlation coefficients (r)			
	r (pH-free P)	r (citrate-free P)	r (enzyme-free P)	r (pH-citrate)
0.00	-0.84945[a]	0.935102[a]	0.644191[a]	-0.86355[a]
0.10	-0.80902[a]	0.67985[b]	0.514679[a]	-0.82572[a]
0.50	-0.75568[a]	0.584846[b]	0.365715[b]	-0.82431[a]
1.00	-0.74505[a]	0.283312[c]	0.228657[b]	-0.69565[b]

Data in the same line followed by the same letter are not significantly different according t-test ($p < 0.05$)

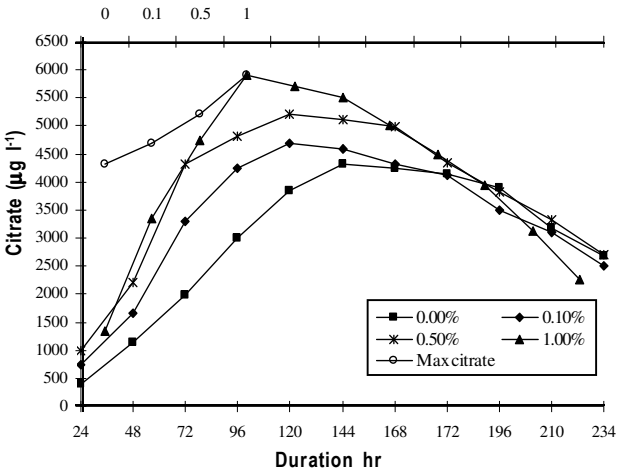

Fig. 3: Production of citric acid during culture of *Aspergillus* sp PS-104 with various concentration of loess

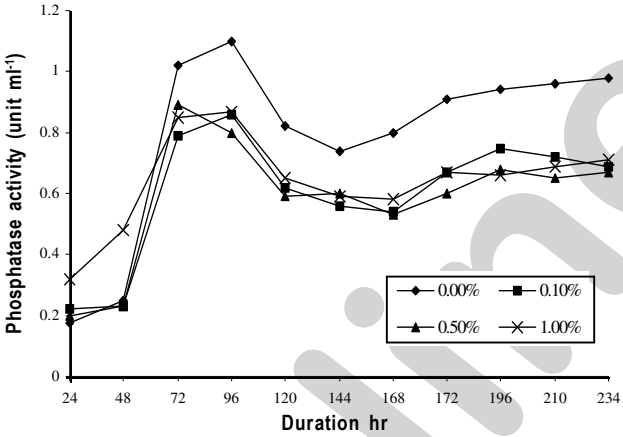

Fig. 4: Phosphatase activity during culture of *Aspergillus* sp PS-104 with various concentration of loess

correlation between the amount of P solubilized and organic acid concentrations (Illmer and Schinner, 1995).

The pH of the broth lowered in absence and with all the concentrations of loess. The trend was similar for all the concentrations. Lowest values were recorded after 144 hr of incubation (Fig. 2). The pH dropped from 6.5 to 2.48 on 6th day followed by a small increase and remained almost constant thereafter, at 1.0% loess. Increase in citrate concentration was recorded during incubation (Fig. 3). However the maximum amount of citrate released was different for various concentration of loess as compared to control. Again, linear relationship was observed in increase of maximum citrate released with different concentration of loess (Fig. 3). Maximum 5910 µg l^{-1} (4th day) of citrate was released at1% loess, as compared to 4325 µg l^{-1} (6th day) in its absence. The citrate concentration increased gradually upto 6 days followed by steady decrease. In the literature, citrate (Cunningham and Kuiack, 1992) and other organic acids are claimed to be responsible for P solubilization. Hence, apart from studying effect of loess on RP solubilization, we derived the correlation coefficient (r) between the P solubilization and citrate production (Table 1). Significant correlation was observed between citrate released and soluble P in absence of loess. Increase in soluble P was also in accordance to decrease in pH, which suggested the role of $[H^+]$ in solubilization mechanism. At 1.0% loess concentration, r (citrate-pH) was relatively less, which suggests that at high concentration of loess, decline in pH was due to other metabolic activities of organism, in addition to citrate production. Citrate concentration also increased during incubation, although maximum value varied with different loess concentrations. The correlation coefficient (r-value) between citrate and soluble P decreased corresponded to increase in loess concentration. This gives the idea that RP solubilization is a complex process, which although governed by citrate here, but other factors also play important role and their cumulative contributions increase with involvement of loess. It appeared that at higher concentration of loess, metabolites other than citrate were also responsible for drop in pH.

The P concentration in the solution did not change according to sigmoid curve type, but showed increases and decreases. So different parameters were detected throughout the incubation after regular intervals, instead of specific time as suggested (Illmer and Schinner, 1995). Increases and decreases in soluble P concentration suggested the formation of some intermediate products and secondary solubilization as earlier hypothesized (Illmer and Schinner, 1995). One of the possibilities for non-sigmoid behavior is re-immobilization of soluble P in insoluble organic and inorganic forms. In soil, phosphatases are responsible for release of soluble P from soluble organic P esters. These released P entertains a possibility of re-immobilization in insoluble inorganic forms (mineralization). Production of phosphatase from aspergilli is well known (Tarafdar *et al*., 1988). Phosphatase activity increased to maximum between 72-96 hr of culture time (Fig. 4). The enzyme activity decreased thereafter up to 6th day followed by increase up to 11th day of incubation. Trend was found to be similar for different concentrations of loess. However, relatively higher amount of phosphatase was released in its absence. Further, partial correlation was observed between P solubilization

and phosphatase activity, in the presence of low amount of loess. Conversely, r (enzyme-soluble P) was low at higher concentrations of loess. This avoided us to draw any actual relationship between the two processes. Virtually, it was observed that, soluble P concentration was high when enzyme production was low (120-172 hr) and vice-versa, which suggest that some of the solubilized P was again precipitated in intermediate forms *i.e.* formation of some intermediate products and secondary solubilization. This process might be repeated several times with different inorganic or organic compounds, making solubilization of inorganic P hardly predictable (Illmer and Schinner, 1995).

Our findings suggest that process of P solubilization by *Aspergillus* sp PS104 is a very complex process, and the complexity of the mechanism increases with the involvement of soil. It has been suggested that the solubilizing ability is not related to organic acid produced but to the nature of organic acid produced (Kang *et al*., 2002). In our findings, P solubilization was related more to H^+ concentration rather than citrate production, the latter perhaps assists in increase of H^+ concentration *Aspergillus* sp PS104 was found to have the capability of solubilizing the RP. A few workers have reported RP solubilization in liquid medium by various fungi (Narsian and Patel, 2000, Kang *et al*., 2002). The use of RP not only compensates for higher cost of manufacturing fertilizers in industry but also mobilizes the fertilizers added to soil. Addition of loess to broth culture enhanced the P solubilization process. Further investigations are needed to focus on the action of *Aspergillus* sp PS104 under *in situ* conditions, in order to access and describe its effect in the rhizosphere.

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