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Studies on the mechanism of solubilization of Indian rock phosphate by *Aspergillus niger* AB100

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A study on the mechanism of solubilization revealed that phosphorus solubilization was attributable both release of organic acids in the broth medium and partially due to enzymatic decomposition of the rock by *Aspergillus niger* AB100. Glutamic acid was the major acid responsible and this was produced by the fungus on utilization of glucose. Other acids produced were citric acid though it was produced in very small amount. Another mechanism responsible for release of phosphorus was the enzyme acid phosphatase which was produced both extracellularly and intracellularly and caused hydrolytic cleavage of the rock. Scanning electron micrographs, petrographic studies and X-ray diffraction studies proved that phosphate is removed from the ore to a considerable extent by *Aspergillus niger* AB100. From the above mentioned mechanism, it was found that about 72.25% of phosphorus solubilized from rock phosphate which was estimated titrimetrically.

Keywords: Solubilization, organic acids, *Aspergillus niger* AB100, phosphatase.

Introduction

Phosphorus, one of the major nutrients for crops is involved in many essential physiological processes including cell division, photosynthesis, breakdown of sugars, energy transfer and nutrients transfer in microorganisms[1]. At the same time the role of phosphorus in expression and maintenance of genetic materials is also well established[2]. Plants obtain phosphorus from the soil in the forms of HPO_4^{2-} and $H_2PO_4^{-3}$. However, the major portion of the applied phosphorus gets fixed into unavailable forms in the soil resulting in of 10% becoming available to plants[4]. The sink of fixed phosphorus can be obtained biologically using the mineral phosphate solubilizing (MPS) microorganisms[5]. Many studies have indicated that, inoculation of soils with MPS microorganisms has resulted in increased phosphate uptake and economic viability in crop developments[5,6]. Many evidences have accumulated supporting solubilization of phosphate by microorganisms[7,8]. Most of the reports of solubilization of insoluble phosphate by microorganisms in liquid culture and in soil suggest that organic acid metabolites produced extracellularly by microorganisms are sole responsible for solubilization of phosphate compounds[9–11]. It was observed that these fungi which decreased the pH of the medium, maximum effected better solubilization of phosphate. However, reports are presently indicating that certain microorganisms solubilize phosphate in alkaline range[12–16]. Several processes work in conjugation with each other for phosphate solubilization[17,18]. Organic acids play a major role as they act as cultivating agents as well as have a direct acidifying effect on the surrounding medium[19]. Rose (1957) concluded that *Aspergillus niger* could able to use ferric phosphate into solution by producing H_2S gas[20]. Since then many authors have demonstrated that formation of acid, alkalinity, chelating agents, exchange reactions, enzymatic reactions are responsible for solubilization of phosphate. Asca (1988) showed that there was a drop in pH when a medium containing equimolar concentrations of NH_4^+ and NO_3^- as sources of nitrogen which ultimately led to phosphate solubilization[21]. Also some reports suggest that cultures supplied with an equimolar concentration of glucose and NH_4^+, stoichiometric coupling of H^+ excretion takes place with NH_4^+ uptake. This causes generation of inorganic acids and acidification of the medium[22].

Thus, an attempt was made to study the role of organic acids, enzymatic dissolution and other factors in the solubilization process. Structural alterations of the rock before and after the solubilization by *Aspergillus niger* AB100 was also studied by electron microscope, pictographic analysis and X-ray diffraction.

Materials and methods:

Collection of rock phosphate ore: Samples of rock phosphate ore were obtained from Rajasthan State Mines and Mineral Ltd., Udaipur and crushed in a ball mill to make fine granules. It was further passed through a sieve 200 mesh. Particles were collected for rock phosphate solubilization experiments.

Microorganism: The parent strain *Aspergillus niger* X1 was isolated from North Bengal soils and exposed to ethyl methane sulfonate (EMS) for 2 h (0.05 to 0.2 *M*) and X-rays (Co^{60}: 10–50 Krad). The maximally solubilizing mutant *Aspergillus niger* AB100 was subcultured on czpek dox agar slants for several generations. They were then transferred to slants of malt and yeast extract agar for solubilization experiments.

Medium and culture conditions: The medium used for solubilization of rock phosphate ore by the phosphate solubilizing microorganisms consisted of $NaNO_3$, 0.4%; KCl, 0.1%; KH_2PO_4, 0.03%; yeast extract, 0.1%. This was sterilized at 121°C for 15 min. A 4.0% glucose solution was sterilized separately and added to the medium. Riboflavin, 5.0 μg/ml; biotin, 1.0 μg/ml; cysteine, 0.75 μg/ml; glutamic acid, 1.0 μg/ml and $FeSO_4.7H_2O$, 10 μg/ml was added to the fermentation medium aseptically. The pH of the medium was adjusted to 4.0 and surface culture fermentation was carried out using 250 ml conical flasks each containing 100 ml of fermentation medium (FM) and 0.1% rock phosphate ore of 200 mesh size. The flasks were incubated at 28°C for 7 days. After incubation, the mycelial mat was separately from the filtrate and soluble phosphate was determined.

Determination of phosphorus: Soluble phosphate was determined titrimetrically by taking a fixed volume of the filtrate and precipitating with 10% ammonium molybdate and dissolving it in (N/10) NaOH and titrating with (N/10) HCl using phenolphthalein as indicator. From the volume of alkali consumed percentage P_2O_5 can be calculated.

1 ml (N) NaOH≡0.003087 g; P_2O_5≡0.00139 g phosphate

To calculate the amount of phosphorus solubilized and the contents of the spent media minus that of the uninoculated control was considered. Also, the amount of phosphorus provided in the form of KH_2PO_4 (0.03%) for growth of the microorganism was subtracted[23].

Determination of organic acids: Organic acids produced in the culture filtrate were analysed by paper chromatography. Samples were spotted on Whatman chromatographic paper No.1 and the separation was carried out using a solvent system of butanol, acetic acid and water in the ratio 4:1:1. At the end of the run, the spots were detected by spraying with glucose-aniline reagent and then heated at 125°C for 5 min. Identification was performed by measuring and compairing R_f values with standard samples. Quantitative analysis was done by measuring total acidity in respect of oxalic acid by using N/10 NaOH and phenolphthalin as indicator[24].

Assay of enzymatic activity: The culture filtrate and the mycelium were tested to detect the process of phosphatase enzyme which can cleave phosphate compounds by hydrolysis.

Measurements of endogenous enzymes: The mycelia mat obtained after filtration of the fermentation broth was washed with distilled water. The mycelium was pressed by hard close cloth and weighed (damp weight). A quantity of quartz sand (twice the damp weight of the mycelium) was added to the mycelium in a chilled mortar and grinded in the cold for 5 to 10 min. Sodium acetate and acetic acid buffer (0.1 N, pH 5.4) was added the extract. Solubilized enzymes and the extract centrifuged at 10,000 rpm for 15 min in a HIMAC centrifuge at 4°C. The final supernatant was used as the endogenous source of enzymes[25].

Measurements of endogenous enzymes: The culture filtrate was collected by passing *Aspergillus niger* AB100 cultures after 8 days of solubilization experiments through Whatman No. 4 filter paper. The filtrate was collected in 250 ml volumetric flask and the volume made up to the mark with double distilled water. Phosphatase activity was measured by the method described below:

Phosphatase assay: 0.1 ml of enzyme solution was inoculated at 37°C for 30 min with 2 ml buffer (0.1 N sodium acetate and acetic acid buffer, pH 5.4) and 0.5 ml paranitrophenyl phosphate (30 mM) which acted as the substrate. The reaction was terminated by adding 1(N) NaOH and the absorbance noted at 420 nm. The absorbance was com-

pared with a calibration curve drawn for standard *p*-nitrophenol solution (100 µg/ml). The entire assay was carried out in duplicates with corresponding controls (without substrate). Phosphatase activity was expressed in µmol of *p*-nitrophenol liberated/minute/ml of culture filtrate at 37°C. One unit of phosphatase activity is defined as the release of 1 µmol of *p*-nitrophenol/minute[26].

Structural alterations of rock sample: In view of the structural alterations of the rock, Scanning Electron Microscopy (SEM), pectrographic examination and X-ray diffraction were performed.

Preparation of samples for SEM: Rock samples before and after fermentation of –200 mesh size were taken. The grains were then mounted on a stub with double sides adhesive tape and silver paste was coated slowly with a very thin (2–5 nm) layer of gold (Boyde, 1971) in a sputtering unit. SEM was done in LEO (UK) SEM, Model No. S-440[27].

Preparation of sample for petrographic analysis: This study was performed by Polarizing Microscope (PM) model Ortholux II, Pol-BK, Leitz, Germany. Samples of both treated and untreated ores were fixed on glass slides and then observed through the PM. Two types of lens were used, crossmicol and plane polarized[28].

X-Ray diffraction: X-Ray diffractograms were taken in a Philips PW 1730/10 generator fitted with a gonumeter PW 1015/81, a preparational counter, a PW/1710 diffractometer control with computerized arrangements for recording. Nickel filter Cuk radiation was used. The tubes were run at 30 KV 20 mA.The 'd' volumes were directly found from a cleast 20 vsd. JCPDS curves were used for identifying the phase present[29].

Statistical analysis: Values were expressed as mean ±SEM, where n = 6.

Results

Determination of organic acids: The major acid responsible for solubilization was glutamic acid reported by other markers. The presence of other acids like acetic acid and citric acid has also been observed.

Quantitative estimation of organic acids: Total acidity was measured in terms of oxalic acid. It was observed that the acidity increased with progressive days of incubation (Fig. 1).

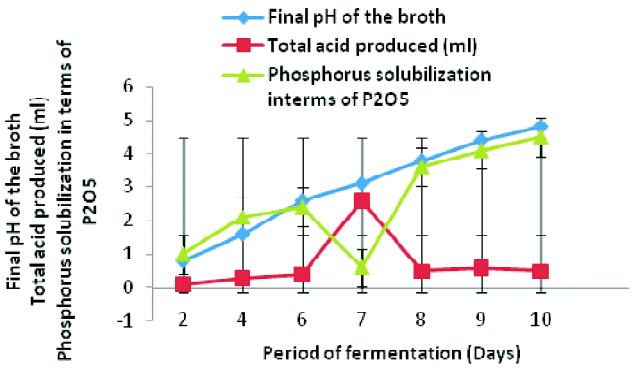

Fig. 1. Relation of total acid produced with pH and phosphorus solubilization (Values were expressed as mean ±SEM, where n = 6).

Relation of phosphatase enzyme with phosphorus solubilization: Phosphatase enzyme activity of the fungus was estimated during the days of incubation. Linear correlation between the phosphatase activity and the fungal growth was observed similar to the literature available[29,30]. Also a negative correlation between pH and the acid phosphatase produced by the mycelia was observed. Since fungi are the major producers of acid phosphatase, these enzymes are adopted to acidic environment. A significant correlation between the phosphorus solubilized and phosphatase activity has also been observed (Fig. 2)[31,32]. These enzymes are responsible for hydrolytic cleavage of the rock and acid fertility to soil[33,34].

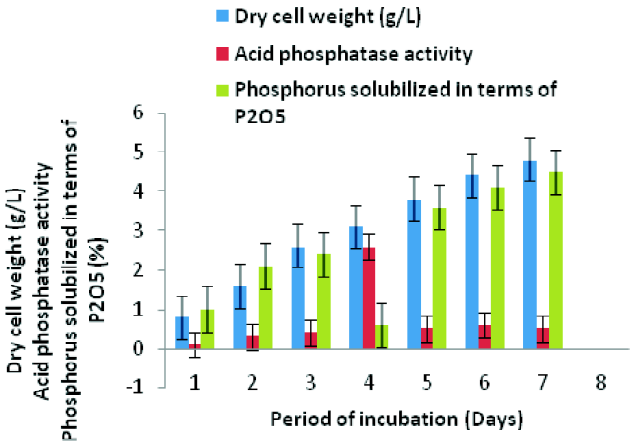

Fig. 2. Total acid phosphatase activity in relation to cell growth and phosphorus solubilized on different days of incubation (Values were expressed as mean ±SEM, where n = 6).

Scanning Electron Microscopy: Particles of untreated Rajasthan rock phosphate were irregularly shaped and contained crystalline components (Fig. 3). After treatment, the microscopy of the rock changed to electron transparent geometrical shaped particles with smooth edges. The smaller components were not visible after treatment (Fig. 4). Phosphorus solubilization was increased with the advancement of incubation period which as seen in Figs. 1 and 2. That led to increment of the erosion of the ore. Cracks could also be observed in the particles.

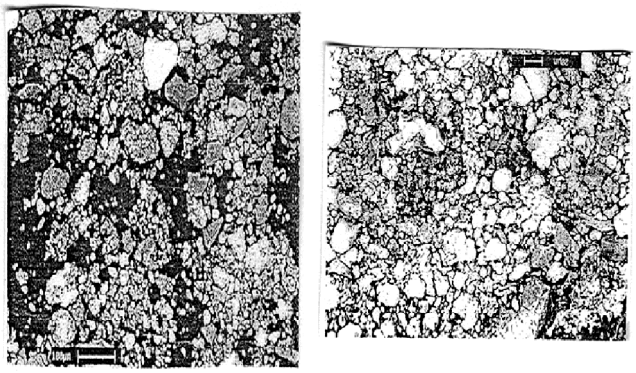

Fig. 3. SEM of untreated rock phosphate.

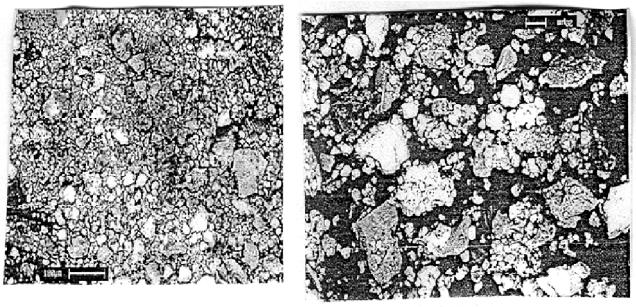

Fig. 4. SEM of treated rock phosphate.

Petrographic examination: Petrographic analysis reveals that Rajasthan rock phosphate contains a considerable quantity of apatite (centrally located apatite grains colored grayish white along with small scattered grain, Fig. 5). Weathering of Rajasthan rock phosphate resulted in smooth and rounded edges crystals. Cubical shaped impressions were formed which scattered light in all directions (Fig. 6). Also cracked and crevices were clearly visible in all particles.

X-Ray Diffraction analysis: By comparing X-ray diffraction patterns of the initial apatite with those of the product

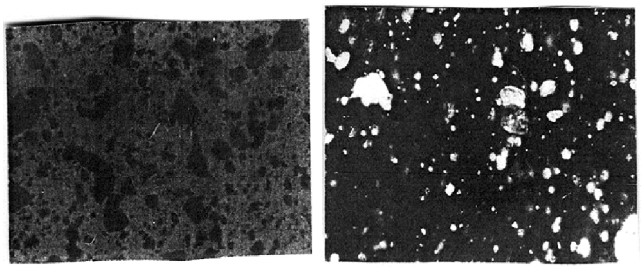

Fig. 5. Petrographic study of untreated rock phosphate (X250).

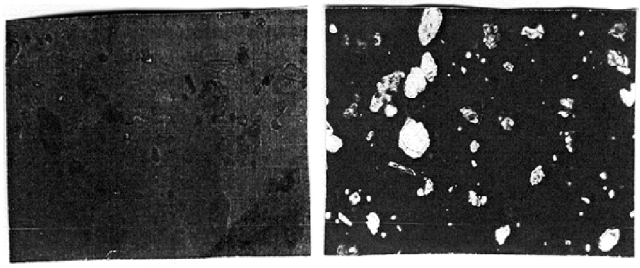

Fig. 6. Petrographic study of treated rock phosphate (X250).

obtained after incubation it was observed that chateristic lines at

D = 4.1775, 3.992, 3.8225, 3.6493, 3.3920, 3.2995, 3.1399, 3.0253, 2.9889, 2.8376, 2.7733, 2.7367, 2.6700, 2.5934, 2.4275, 2.2717, 2.2656, 2.2317, 2.1252, 2.1133, 2.0786, 2.0429, 2.0126, 1.9220, 1.9201, 1.8975, 1.8681, 1.8260, 1.8158, 1.7602, 1.7254, 1.6625, 1.6300, 1.5950, 1.5594, out of which lines of apatite at d = 3.992, 3.8225, 3.6493, 3.3920, 2.9889, 2.8376, 2.7367, 2.4275, 2.2717, 2.2656, 1.8975, 1.8260 disappeared completely. Also, the intensity of the peaks decreased. Thus, a structural alteration took place.

The solubilization of rock phosphate by *Aspergillus niger* AB100 involved the interplay of various mechanisms. The production of low molecular weight organic acid by *Aspergillus niger* AB100 is one of the major factors responsible for solubilization. These organic acids bring about phosphorus dissolution by mechanisms involving chelation and exchange reactions. However, other factors like phosphatase enzyme are also responsible for increasing phosphorus in solution. These enzymes are most active in low pH, so under conditions of acidic pH due to production of organic acids, phosphatases dephosphorylate the inorganic acid and organic phosphates of the rock.

Discussion

Rock phosphate is the cheapest and abundant source of phosphatic fertilizer, aboundantly available but due to its less solubility available in water, it is not economically viable in agrochemical industries. Phosphate solubilizing microorganisms has emerged as eco-friendly and cheep method. Omar (1997) isolated 36 fungal species from soil and tested their ability to solubilize rock phosphate. Most of them were proved to be non-solubilizer for rock phosphate except *Aspergillus niger* and *Penicillum citrium*[35]. To increase the soil fertility, the phosphate solubilizing fungus *Penicillum oxalicum* was isolated from rhizophore soil of rock phosphate mine landfills and examined for its solubilizing efficiency for rock phosphate. The results showed that this fungus effectively solubilize rock phosphate in Pikovskaya's medium and liberated higher amount of phosphorus[36]. Sane and Mahta (2015) tested 30 different fungal strains for rock phosphate solubilization study using 5% Senegal rock phosphate as a source of phosphorus in Pikovskayas's medium, among which only three strains exhibited rock phosphate soulbilizing activity[37]. A total of 359 fungal strains were isolated by a group of scientists from 150 rhizosphere soil samples of haricot bean, faba bean, cabbage, tomato, sugar cane, among which 167 belonging to *Aspergillus* sp. (55.69%), *Penicillum* sp. (23.35%) and *Fusarium* (9.58%) exhibited rock phosphate solubilizing capacity with solubilization index (SI) ranged from 1.10 to 3.50[38]. Gizaw *et al*. (2017) isolated a soil fungus *Tricosporon beigelii* B, with *Rhodotrula aurantaca* A[39]. Very recently, a study was carried out to examine the strategies for microbial solubilization of rock phosphate using Eppawala rock in Sri Lanka. *P. fluorescens* was proved to be an effective microorganism in this connection[40].

Conclusion

The present investigation focused on the solubilization of Indian rock phosphate from ore supplied by Rajasthan State Mines and Mineral Ltd. By *Aspergillus niger* AB100 partially attributed by phosphatase enzyme and glutamic acid. About 72.25% phosphorus was solubilized from rock which estimated titrmetrically and the events were visualized morphologically by electron microscopy, pectrographic studies and X-ray diffraction studies.

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