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ORIGINAL ARTICLE

Solubilization of Different Inorganic Phosphates by *Aspergillus niger* and *Penicilium oxalicum*

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ABSTRACT
Aspergillus niger and Penicillium oxalicum were isolated from the rhizosphere soil of tomato. Both showed immense phosphate solubilization of Pikovskaya's agar (PVK) plate. The phosphate source used in PVK plate was Tri-calcium phosphate. The fungi were tested for the solubilization of five phosphate sources like Single Super Phosphate (SSP), Rock Phosphate(RP), Aluminum Phosphate(Al_2PO_4), Zinc phosphate ($ZnPO_4$) and Tri-calcium phosphate (TP). Parameters like biomass, available phosphate, pH, titrable acidity and gluconate estimation were determined. Biomass increased with tri-calcium phosphate as phosphate source for both the fungi. The pH of the culture filtrate varied from 2.4-3.0 except in SSP were the pH dropped as low as 1.75 and 1.74 for Aspergillus niger and Penicillium oxalicum respectively. The titrable acidity keeping H3PO4 as acid produced was found to be highest with Al_2PO_4 for Aspergillus niger and with $ZnPO_4$ for Penicillium oxalicum. There was no change in gluconate produced by Aspergillus niger. The highest gluconate produced by Penicillium oxalicum was with $ZnPO_4$ and lowest with Al_2PO_4. From the fall in pH it is concluded that the solubilization of phosphate is mainly due to the production of organic acids and gluconic acid is found to be one of the organic acids produced by these fungi.
Key words: *Aspergillus niger, acidification, Penicillium oxalicum, phosphate solubilization and phosphate solubilizing fungi.*

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INTRODUCTION

One of the most important nutrients required for plants is phosphorus (P). The phosphorus present in soil, approximately 95-99% is in the form of insoluble phosphates which cannot be utilized by plants. Phosphorus availability in soil is increased by addition of fertilizers. But these fertilizers are not completely taken up by the plants; a part of it is converted to insoluble forms and remains in soil [1].

The concentration of soluble phosphorus in many soils is very low when compared to other nutrients available in soil. Phosphorus is available in milli molar quantity whereas the other nutrients are available in micro molar quantities. Most of the inorganic phosphates in acidic soils are salts from iron or aluminum, whereas calcium phosphates are the predominant forms in neutral or calcareous soils.

Phosphorus is important for the functions of many important enzymes that are involved in metabolic pathways. Therefore play a significant role in plant metabolism which is reflected on the pants yield.

It is estimated that about 98% of Indian soils contain insufficient amounts of available phosphorus, which is necessary to support maximum plant growth. The uptake of phosphorus by the plant is only a small fraction of what is actually added as phosphate fertilizer [2]. The remaining phosphorus is later converted to insoluble forms of phosphates and lost in the soil due to adsorption, precipitation, or conversion to organic phosphates [3].

Filamentous fungi, *Aspergillus* sp and *Penicillium* sp are mainly used for solubilization of phosphate under in vitro conditions. The phosphate solubilization ability is generally due to the release of organic acids, decrease in pH. The organic acids increase the P in the soil through chelation of metal ions linked with the phosphate [4].

The solubilization of phosphate can be due to several processes working in conjunction with each other that is organic acids can act as chelating agents and have acidifying effect on the surroundings. Because of the multiplicity of effects, it is not surprising that the ability to acidify the surrounding media is only weakly correlated with the ability to solubilize inorganic phosphate [5& 6]. It has also been observed that the organic acids form soluble complexes with metal ions like Ca, Al and Fe, in turn releasing the phosphate bound to metal ions [7].

Fungi perform better solubilization of phosphate in acidic soil conditions. *Aspergillus* sp, *Penicillium* sand yeast have been reported for solubilizing several forms of inorganic phosphates. Better than bacteria, fungi are reported to possess greater ability to solubilize insoluble phosphates[1]. Several soil fungi, particularly those belonging to the genera *Penicillium* and *Aspergillus* possess ability to bring insoluble soil phosphates into soluble forms by secreting weak organic acids such as formic, acetic, propionic, lactic, glucolic, fumaric and succinic [8].

In the present study, phosphate solubilizing fungi were isolated from the rhizophere soil of tomato. The isolated fungi were examined for its ability to solubilize different sources of inorganic phosphates.

MATERIALS AND METHODS:

Isolation and identification of phosphate solubilizing fungi

The Fungi were isolated from the rhizosphere soil by plating on PVK agar (Pikovskaya's). The isolates formed a clear halo zone after 4 days of incubation and it was maintained PDA (Potato Dextrose Agar). The isolates were identified and further confirmed by sending these cultures to NFCCI, Pune.

Media with different Phosphate sources:

The basal media PVK was used to which 5g/ltr concentration of five different phosphates were prepared separately. The 5 phosphate sources used were Tri-calcium phosphate (TP), Rock Phosphate (RP), Single super phosphate(SSP), Aluminum phosphate(Al_2PO_4) and Zinc phosphate($ZnPO_4$) and the pH of the media were set to 6.5. The media were inoculated with $4x10^8$ spores/ml of *Aspergillus niger* and *Penicillium oxalicum* and incubated for 3 days at 27 °C at 100 rpm.

Detection of fungal biomass, pH of the filtrate and available phosphate estimation:

After three days, the culture filtrate was obtained by filtration using Whatman filter paper no 1. The fungal mass which remained after filtration was dried in hot air oven at 60°C for until there was no water content present. The pH of the culture filtrate was also noted after filtration using standard pH meter [9].

The soluble P concentration was estimated as described by Murphy & Riley [10] where 1ml of culture filtrate and 3ml of the acid molybdate reagent was added and read at 830nm using UV-Vis spectrophotometer (Shimadzu UV-1800). The amount of phosphate solubilized reported was after the deduction of P released by autoclaving.

Detection of Titrable acidity

Titrable acidity (TA) was estimated by titrating 1 ml of the culture supernatant against 10 mM sodium hydroxide in presence of phenolphthalein [9]. The procedure was repeated thrice for each sample and the average of the three was used for calculation.

Gluconate estimation

The concentration of gluconate produced was estimated as described by Nenwani *et al*. [11] with slight modification. The gluconate was estimated titrimetrically against 0.5 M Ethylenediaminetetraacetic acid with pH of 8 using 0.05% of Erichrome T dye prepared using ethanol.

Statistical Analysis:

The data obtained was in triplicates and the data was analyzed for standard deviation using Microsoft Excel software.

RESULTS AND DISCUSSION

Identification of the fungal cultures

The isolates were identified as *Aspergillus niger* and *Penicillium oxalicum* by preparing wet mounts with lacto phenol cotton blue and confirmed by NFCCI, Pune. *Aspergillus niger* produced colonies that were composed of white or yellow felt which is covered by dark asexual fungal spores. The mycelium were septate and transparent. The conidiophores were arranged on a globular vesicles and each of these globular vesicles are completely covered with biserate philide which are projected from the conidiophore (Figure-1). The mycelium of *Penicillium oxalicum* were transparent and multinucleated, highly branched mycelium. The conidiophores were arranged in a form of chain on a philide. The spores were green in colour and the colony formed initially was white in colour and after formation of spores they were green in colour (Figure-2).

Biomass

The biomass weighed showed a large variation. The high dry mass for both the fungi were observed with PVK broth supplemented with tri-calcium phosphate and *Aspergillus niger* showed higher biomass (1.26g) when compared to *Penicillium oxalicum* (0.27g). The lowest biomass for *Aspergillus niger* was seen with SSP, whereas for *Penicillium oxalicum* it was seen with RP (Figure-3).Tri-calcium phosphate as a source, produced higher biomass for both *Aspergillus niger* and *Penicillium oxalicum*, indicating that the fungi were able to solubilize the phosphate and also utilize enough phosphorus for its own growth. Similarly Whitelaw et al [9] had reported that the fungi *Penicillium radicum* biomass with tri-calcium phosphate was 0.316g whereas the biomass with Al_2PO_4 was not determined.

Available phosphate

The highest available phosphate for *Aspergillus niger* was observed with $ZnPO_4$ (4.26mg/ml) and lowest with Al_2PO_4 (0.93mg/ml). Similarly for *Penicillium oxalicum* the highest available phosphate was observed with $ZnPO_4$ (3.224mg/ml), whereas the lowest was observed with SSP (0.17mg/ml) (Figure-4). Nenwani et al [11] had reported that the highest P solubilization was on day 18 with 0.662 mg per ml of the filtrate with Tri-calcium phosphate as the source. Pradan and Shukla [1] have used *Aspergillus fumigatus* to solubilize tri-calcium phosphate using PVK medium, the P solubilized in 4 days to give 0.48mg/ml of P. whereas the *Penicilium sp* solubilized the phosphate in 3 days giving 0.275 mg/ml. when they used rock phosphate as substrate, *Aspergillus fumigatus* released only 0.058 mg/ml of P in culture medium after 7 days of incubation. In the present experiment, *Aspergillus niger* released 1.879 mg/ml of soluble P with tri-calcium phosphate on day 3 and 2.859 mg/ml of soluble P with rock phosphate on day 3. *Penicillium oxalicum* released 3.029 mg/ml of soluble P on day 3 with tri-calcium phosphate and 1.369 mg/ml of soluble P with rock phosphate on day 3. This efficient solubilization of rock phosphate can be due to the significant decrease in pH.

Effect of pH, titrable acidity and gluconate concentration on phosphate solubilization

A significant decrease in pH was observed in all the five filtrates for both the organisms. The lowest pH was observed in SSP for both the cultures, (*Aspergillus niger*-1.75 and *Penicillium oxalicum* -1.74), followed by Tri-calcium phosphate (*Aspergillus niger*-2.68 and *Penicillium oxalicum* -2.51). The pH of *Penicillium oxalicum* with Rock phosphate was slightly higher when compared to *Aspergillus niger* with rock phosphate and also when compared with other phosphate sources (Figure-5). Pradan and Shukla [1]have reported that the solubilization of the phosphate was accompanied by the drop in the pH of the medium, with *Aspergillus fumigatus* the pH decreased from 7.0 to 4.0 in 4 days and with *Penicillium sp* the pH decreased from 7.0 to 4.7 in 3 days.

The titrable acidity was estimated using the filtrate assuming that H_3PO_4 was the major acid produced. Highest acidity for *Aspergillus nige*r was observed with Al_2PO_4 (0.70%) whereas lowest was observed with RP (0.27%). And highest acidity for *Penicillium oxalicum* was seen with $ZnPO_4$ (0.348%) and lowest with RP (0.078%) (Figure-5). The presence of H_3PO_4 can be one of the reasons for the drastic decrease in pH of the culture filtrate.

The gluconate concentration with *Aspergillus niger* did not show much variation as the value was similar for filtrates of Tri-calcium phosphate, SSP and Al_2PO_4 ($2.94X10^{-4}$ g%) whereas similar value was obtained for filtrate of Rock phosphate and $ZnPO_4$ ($1.96X10^{-4}$ g%). But the gluconate concentration varied in *Penicillium oxalicum*, the highest gluconate concentration was observed with SSP ($2.3539\ X10^{-4}$ g %) whereas the lowest with Al_2PO_4 ($0.7846\ X10^{-4}$ g%)(Figure-5).

The substrate SSP showed drastic decrease in pH, a fair amount titrable acidity and gluconate production, but the soluble phosphate available from this substrate is fairly low when compared with the substrate $ZnPO_4$, which gave the highest amount of soluble phosphate from both the isolates. This may be due to the complexity of SSP. For *Aspergillus niger*, the best phosphate substrate was $ZnPO_4$, followed by rock phosphate and tri-calcium phosphate, whereas *Penicillium oxalicum,* the best phosphate substrate was found to be $ZnPO_4$, followed by Tri-calcium phosphate and rock phosphate.

The drastic decrease of the pH in the media showed that the solubilization of the inorganic phosphate is by production of organic acids and by the forms of P present as phosphoric acid. The amount of gluconate produced shows that it is not enough to reduce the pH of the medium to such low levels. This shows that there are other types of acid produced which are responsible for fall in pH. Similarly Nenwani *et al*.[11] observed that gluconic acid produced is not sufficient enough to reduce the pH of the culture medium. Fungi like *Aspergillus flavus*, *Aspergillus niger*, *Penicillium* are known to produce organic acids other than gluconic acid like citric acid, oxalic acid, succinic acid etc, *canescens* [12] hence the P solubilization can be due to the drop in pH by production of organic acids. Calcium phosphate minerals are known to be solubilized by the production of gluconic acid by *Erwinia herbicola* [13], *Penicillium sp.*[14], and *Aspergillus niger* [15]. It has also been involved in the solubilization of rock phosphate by *Penicillium*

variabile [16]. Wenzel *et al*. [17] reported that several potential mechanisms for phosphate solubilization, which include modification of pH by secretion of organic acids and protons or cation dissociation.
Whitelaw *et al*.[9] had reported that the main mechanism for phosphate solubilization by the fungi *Penicillium radicum* is by acidification. It was seen that below pH 5 the solubility of Ca, Al phosphates increased as the pH of the medium decreased. The same was also reported by Nath *et al* [18].
Apart from acidification, other mechanism for phosphate solubilization is by complexation of the cations associated with insoluble phosphates by organic acids. The organic acids like gluconic acid which is having an alpha-hydroxyl acid structure is able to chelate metal ions like Ca^{+}, Al^{+} and Zn^{+} to form calcium gluconate, aluminum gluconate and zinc gluconate. Most of the reports state that calcium phosphates are dissolved by acidification. Hence, any microorganism that acidifies the surrounding medium will show some level of phosphorus solubilizing activity. The proton substitution reactions are driven by microbial production of organic acids, represented generically by the equation [19].

$$(Ca_2+)_m(PO_4^{3-})_n + (HA) = (H^+)\,(PO4^{3-}) + (Ca^{2+})\,(A^-)$$

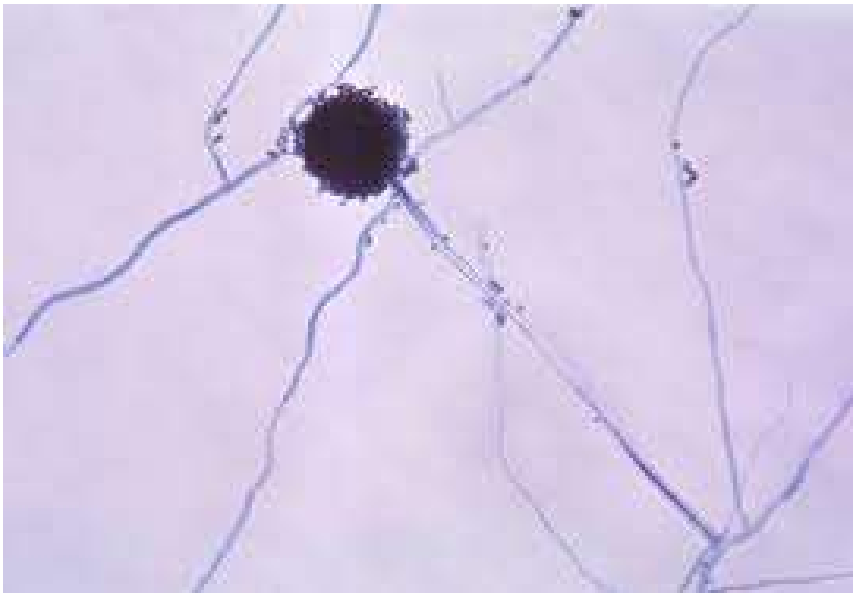

Figure: 1 A typical *Aspergillus* Sporangia

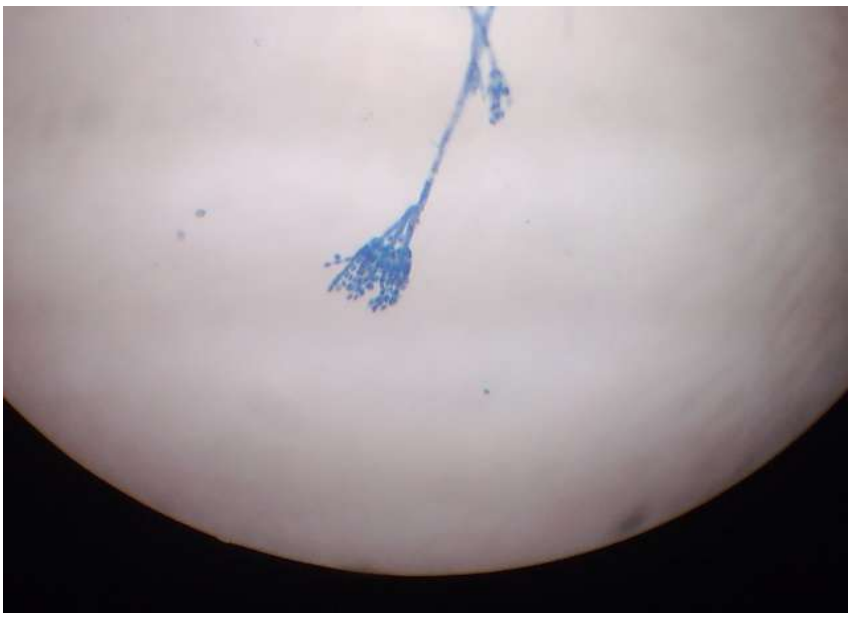

Figure: 2 A typical *Penicillium* Sporangia

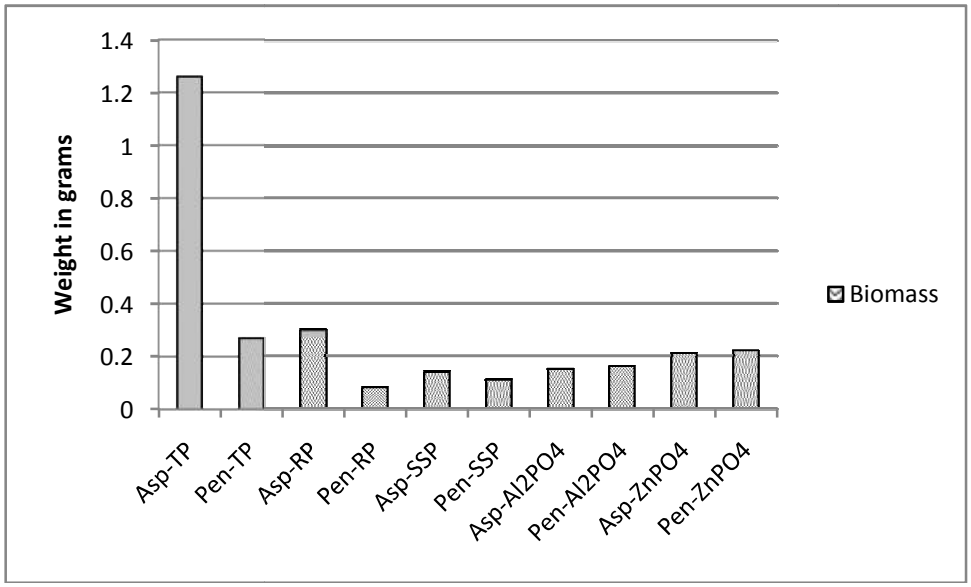

Figure: 3 Biomass of *Aspergillus niger* (Asp) and *Penicillium oxalicum* (Pen) with different phosphate sources. TP-Tri-calcium phosphate, RP- Rock Phosphate, SSP- Single super phosphate, Al2PO4-Aluminium phosphate and ZnPO4-Zinc phosphate.

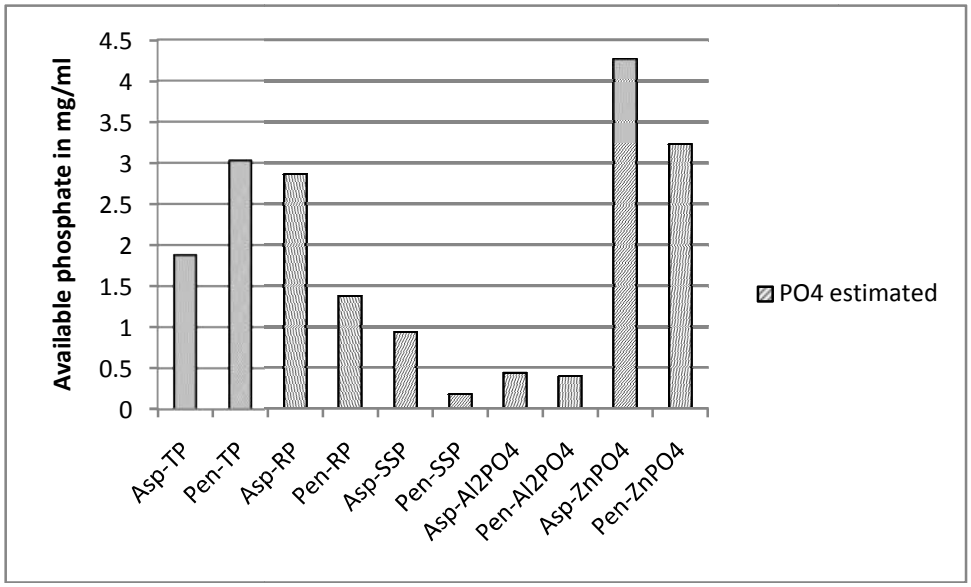

Figure: 4 Soluble phosphate released from different sources by *Aspegillus niger* (Asp) and *Penicillium oxalicum* (Pen)**.** TP-Tri-calcium phosphate, RP- Rock Phosphate, SSP- Single super phosphate, Al_2PO_4-Aluminium phosphate and $ZnPO_4$-Zinc phosphate.

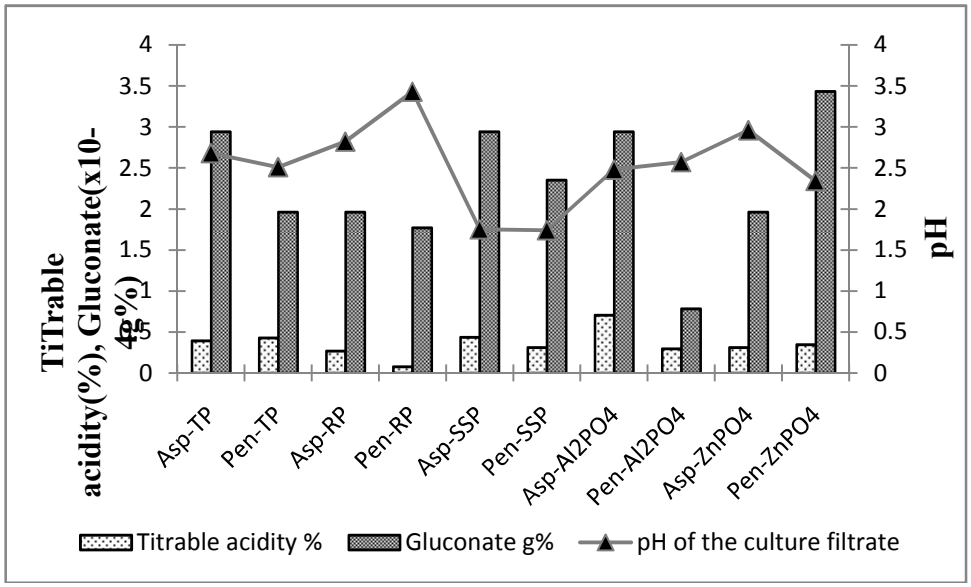

Figure: 5. pH, Titrable acidity and Gluconate produced by *Aspergillus niger* (Asp) and *Penicillium oxalicum* (Pen) with different phosphorus substrate**.** TP-Tri-calcium phosphate, RP- Rock Phosphate, SSP-Single super phosphate, Al2PO4-Aluminium phosphate and ZnPO4-Zinc phosphate.

CONCLUSION

From the above study, *Aspergillus niger* and *Penicillium oxalicum* were able to solubilize tri-calcium phosphate, rock phosphate, single super phosphate, aluminium phosphate and zinc phosphate. Solubilization was best with Zinc phosphate as a substrate for both the isolates. For *Penicillium oxalicum* the second best phosphate source was found to be tri-calcium phosphate and for *Aspergillus niger* rock phosphate was found to be the second best. As both the organisms have the ability to solubilize the different inorganic phosphates, these can be used as phosphate solubilizers and in combination with chemical fertilizers and bio fertilizers

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