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Full Length Research Paper

Solubilization of inorganic phosphates by fungi isolated from agriculture soil

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Most agricultural soils contain large reserves of phosphorus (P), a considerable part of which accumulates as a consequence of regular applications of P fertilizers. However, a greater part of soil phosphorus, approximately 95–99% is present in the form of insoluble phosphates and hence cannot be utilized by the plants. In the present study fungal strains isolated from agriculture soil, having potential to solubilize insoluble inorganic phosphates were characterized. Two fungal isolates were tested for their tricalcium phosphate (TCP) solubilization efficiency in both solid and liquid medium. Isolates were identified as *Aspergillus* sp. and *Penicillium* sp. depending upon their colony morphology and microscopic studies. Phosphate solubilization was related to pH decrease caused by growth of fungus in medium containing glucose as carbon source. *Aspergillus* sp. solubilized 480 g/ml of phosphorus, while *Penicillium* sp. solubilized 275 g/ml of phosphorus from 0.5% tricalcium phosphate after 4 and 3 days of growth respectively. Both the strains show diverse levels of phosphate solubilization activity in liquid broth culture in presence of various carbon and nitrogen sources. Drop in pH during growth was more prominent in absence of TCP in the liquid medium. This indicates that absence of soluble P in media induces the acid production. Phosphate solubilizing microorganisms convert insoluble phosphates into soluble forms generally through the process of acidification, chelation and exchange reactions. Thus such microorganisms may not only compensate for higher cost of manufacturing fertilizers in industry but also mobilizes the fertilizers added to soil.

Key words: Tricalcium phosphate, phosphate solubilization, *Aspergillus, Penicillium.*

INTRODUCTION

Phosphorus is one of the major nutrients, second only to nitrogen in requirement for plants. A greater part of soil phosphorus, approximately 95–99% is present in the form of insoluble phosphates and cannot be utilized by the plants (Vassileva et al., 1998). To increase the availability of phosphorus for plants, large amounts of fertilizer are being applied to soil. But a large proportion of fertilizer phosphorus after application is quickly transformed to the insoluble form (Omar, 1998). Therefore, very little percentage of the applied phosphorus is available to plants, making continuous application necessary (Abd Alla, 1994). However, phosphorus deficiencies are wide spread on soil throughout the world and phosphorus fertilizers represent major cost for agricultural production.

Many soil fungi and bacteria are known to solubilize inorganic phosphates (Asea et al., 1988; Illmer and Schinner, 1992). Phosphate solubilizing microorganisms (PSMs) play an important role in supplementing phosphorus to the plants, allowing a sustainable use of phosphate fertilizers. Microorganisms are involved in a range of process that effect the transformation of soil phosphorus (P) and thus are integral component of the soil 'P' cycle. Many bacterial, fungal, yeast, and actinomycetes species capable of solubilizing sparingly soluble phosphorus in pure culture have been isolated and studied (Halder et al., 1991; Abd-Alla, 1994; Whitelaw, 2000; Goldstein, 1986). Application of PSMs in the field has been reported to increase crop yield. Several mechanisms like lowering of pH by acid production, ion chelation and exchange reactions in the

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growth environment have been reported to play a role in phosphate solubilization by PSMs (Halder et al., 1991; Abd-Alla, 1994; Whitelaw, 2000; Goldstein, 1986). Among PSMs, fungi perform better in acidic soil conditions (Ahmad and Jha, 1968). Species of *Aspergillus, Penicillium* and yeast have been widely reported solubilizing various forms of inorganic phosphates (Asea et al., 1988; Whitelaw, 2000).

Fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria (Nahas, 1996). In the present study fungal strains having potential to solubilize insoluble phosphates were isolated. The fungal isolates were checked for the ability to solubilize different insoluble phosphates.

MATERIALS AND METHODS

Microorganisms

Fungal strains were isolated from the rice field soil of Bhubaneswar, Orissa, India after serial dilution of soil solution on Potato Dextrose Agar plates. Isolated, predominant, morphologically distinct colonies were selected, purified by repeated culturing and maintained on PDA slants at 4°C. Isolates were identified by their colony characteristics, spores morphology and microscopic observations. The isolates were checked for phosphate solubilizing ability on Pikovskaya (PVK) medium (Pikovskaya, 1948) incorporated with tricalcium phosphate ($Ca_3(PO_4)_5$). Composition of all mediums used for this study is given in Table 1. Formation of a clear halo zone around the fungal growth after 5 days of incubation indicates phosphate solubilizing ability.

Media and growth conditions

Phosphorus solubilizing ability of fungal strains was tested in six different types of liquid media reported in literature. Compositions of different media are given in Table 1. Rests of the experiments were performed using in PVK medium with 0.5% tricalcium phosphate ('P' 997 g/ml). Flasks were inoculated with 5% (v/v) spore suspension and incubated on a rotary shaker at 30°C for 7 days. Effect of different carbon sources on P solubilization was done with addition of 1% of respective sugar in place of glucose. Similarly for determining effect of different nitrogen source, 0.5% of different nitrogen salts were added to medium instead of $(NH_4)_2SO_4$.

Solubilization of phosphorus from rock phosphate

Rock phosphate sample (RP-140) having P_2O_5 content about 18.8 % was used for the study with *Aspergillus* sp. Quantitative estimation of phosphate solubilization activity was carried out in PVK medium amended with 0.25% (w/v) rock phosphate with other conditions same as for TCP solubilization for a duration of 10 days.

Estimation of phosphorus

Cultures were harvested after different growth periods in order to record the change in pH and concentration of P released in the medium. After centrifugation at 12,000 rpm for 20 min, the pH of the culture medium was measured with a pH meter equipped with a glass electrode. Dissolved phosphate concentration in the culture filtrate was determined by vanado- molybdate method as described in APHA (1995). It was expressed in terms of g/ml phosphorus released in culture medium.

RESULTS AND DISCUSSION

Microorganisms

Out of all bacteria and fungus isolated from the soil only two fungi show significant zone of phosphate solubilization. A clear halo zone was formed around the colonies after 5 days of incubation on solidified PVK medium supplemented with calcium phosphate, indicating phosphate-solubilizing ability of the fungal isolates. These two were selected for further studies. The fungal strains were identified as *Aspergillus fumigatus* and *Penicillium* sp. based upon their colony morphology, spore characteristics and microscopic studies.

A. fumigatus showed blue green surface pigmentation with a suede like surface consisting of a dense felt of conodiophores. Conodiophores are short, smooth walled and have conical shaped terminal vesicle which support a single row of phialides on the upper two third of the vesicle. Conodial heads are green echinulate columnal and uniseriate. Conodia are produced in basipetal succession forming long chains, globuse, green and rough walled.

Penicillium sp. show green colour colonies on PDA plates consisting of a dense felt of conidiophores. Microscopically, conodiophores show branching, and phialides produced in groups from branched metulae, giving brush-like appearance. Conodia are globuse, greenish and smooth.

Solubilization of insoluble phosphates

After confirming the phosphorus solubilizing ability on solid medium, the phosphorus solubilization in liquid medium was carried out. Different researchers have used different media for studying phosphorus solubilization in liquid medium. Therefore we tried to find out which media formulation was best for new isolate *Aspergillus* sp. AYG medium (Halder et al., 1991), PVK medium (Pikovskaya, 1948) and NBRIY medium (Nautiyal, 1999) show maximum P solubilization at rate of 495, 480 and 410 g of 'P'/ml of culture filtrate with resulting final pH of 3.8, 3.95 and 4.1, respectively (Figure 1). Low level of P solubilization was observed in other media. Considering amount of glucose used in medium and corresponding efficacy of P solubilization, PVK medium proved to be most cost effective without compromising the solubilization. Therefore for further studies PVK medium was used.

Phosphate solubilization was accompanied by a decrease in the pH of the medium by both the strains. *Aspergillus* sp. solubilized 480 g/ml of phosphorus from

Table 1. Composition of different media used in this study.

Media components (g/L)	Medium 1 (AYG; Halder et al., 1991)	Medium 2 (Kim et al., 1997)	Medium 3 (Vassilev et al., 1998)	Medium 4 (PVK; Pikovskaya 1948)	Medium 5 (NBRIY; Nautiyal 1999)	Medium 6 (NBRIP; Nautiyal 1999)
Glucose	20	10	100	10	10	10
$(NH_4)_2SO_4$	1	-	-	0.5	0.5	0.1
$MgSO_4.7H_2O$	0.5	0.4	0.2	0.1	0.1	0.25
Yeast extract	0.2	0.5	-	0.5	-	-
KCL	-	-	-	0.2	0.2	0.2
NaCl	-	1	-	0.2	0.2	-
$FeCl_3$	Trace	-	-	-	-	-
$FeSO_4.7H_2O$				0.002	0.002	
$MnSO_4.H_2O$	Trace	-	-	0.002	0.002	-
$MgCl_2.6H_2O$	-	-	-	-	-	5.0
$CaCl_2$	-	0.2	-	-	-	-
NH_4NO_3	-	1.5	0.5	-	-	-
$ZnSO_4$	-	-	0.004	-	-	-
$Ca_3(PO_4)_5$	-	-	-	5.0	5.0	5.0
pH	6.8	7.0	5.0	7.0	7.0	7.0

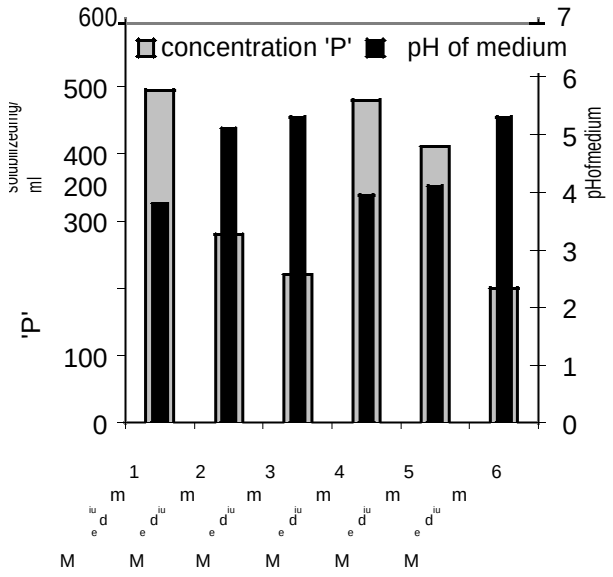

Figure 1. Effect of different media on 'P' solubilzation.

0.5% tricalcium phosphate with decrease in pH from 7.0 to 4.0 in 4 days. *Penicillium* sp. solubilized 275 g/ml of phosphorus in 3 days with pH falling to 4.7 from initial pH of 7.0. The extent of soluble phosphate was negatively correlated with pH of the culture filtrate. *Aspergillus* sp. show much higher drop in pH and simultaneous higher P solubilization when compared to *Penicillium* sp. Treatment of rock phosphate by *Aspergillus* released only 58 g/ml of P in culture medium after 7 days of incubation. It was lower as compared to TCP solubili-zation, which may be due to complexity of the structure.

Phosphorus solubilizing microorganisms are reported to dissolve insoluble phosphates by the production of inorganic or organic acids and/or by the decrease of the pH (Whitelaw, 2000). Most of the previous reports state that calcium phosphates are dissolved by acidification. Therefore, any microorganism that acidifies its external medium will show some level of phosphorus solubilizing activity. In most soils, proton substitution reactions are driven by microbial production of organic acids, represented generically by the equation:

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

There is no stoichiometry in the equation because of the complexity of CaP chemistry and the multiplicity of microbially produced organic acids (HAs) with differing numbers of dissociable protons (Goldstein, 1986).

Factors affecting solubilization of phosphorus

Phosphate solubilization activity of *Aspergillus* sp. was evaluated in the presence of five carbon and seven nitrogen sources, by replacing glucose and $(NH_4)_2SO_4$, respectively of the PVK medium. This strain demonstrated diverse levels of phosphate solubilization activity in the presence of various carbon and nitrogen sources. Production of acids was greatly affected by the nature of carbon sources. Glucose and maltose decreased the pH of the medium to maximum extent and caused highest solubilization of phosphorus, followed by sucrose, xylose and galactose (Table 2). In control flask without any addition of carbon source, some growth did occur due to presence of yeast extract in the medium, but drop in pH and P solubilization was quite low. The solubi-

Table 2. Effect of carbon source on phosphorus solubilization using *Aspergillus* sp.

Condition[a]	'P' (g/ml)	Final pH of medium
Nitrogen source [b]		
$(NH_4)_2SO_4$	411	3.31
Urea	386	5.23
KNO_3	200	4.91
$NaNO_3$	341	5.02
NH_4Cl	232	3.50
$NaNO_2$	239	6.02
Casein	336	3.55
Control[d]	225	5.10
Carbon source [c]		
galactos	257	5.6
sucrose	400	4.8
glucose	433	4.8
maltose	424	4.8
xylose	372	4.9
Control[d]	112	5.8

[a]Strain was grown at 30°C for 7 days in PVK medium.
[b]$(NH_4)_2SO_4$ in the PVK medium was replaced by the nitrogen source as indicated.
[c]Glucose in the PVK medium was replaced by the carbon source as indicated.
[d]Autoclaved, inoculated batch cultures in medium without nitrogen or carbon source served as controls.

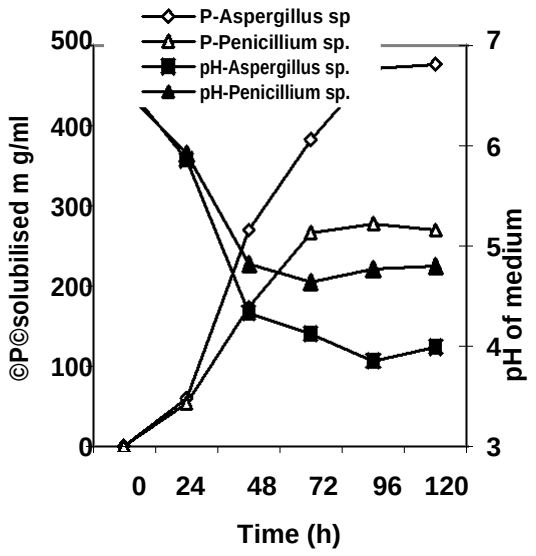

Figure 2. Kinetics of 'P' solubilization from tricalcium phosphate by Aspergillus sp. And Penicillum sp. In PVK medium.

lizing ability of a microorganism is related to its organic acid production, however the nature of the acid produced is also important (Vassileva, 1998).

Nitrogen salts having either ammonium group or nitrate group or both were used as nitrogen source for the study. $(NH_4)_2SO_4$ was found to be best in reducing the medium pH to 3.31 and simultaneous solubilization of 411 g/ml of P, out of all the nitrogen sources used (Table 2). The control flask of the set without any nitrogen source also shows quite a substantial growth, drop in pH and solubilization of P. This was due to the presence of yeast extract and glucose in medium, the former of which is utilized by fungus as nitrogen source. The rest of the nitrogen sources except urea show very marginal increase in P solubilization when compared to control. It was thus observed that *Aspergillus* sp. was able to utilize $(NH_4)_2SO_4$ most efficiently to decrease the pH of the medium for P solubilization. This finding was also evident from effect of different media on P solubilization (Figure 2). AYG medium (Halder et al., 1991) PVK medium (Pikovskaya, 1948) and NBRIY medium (Nautiyal, 1999) show maximum P solubilization and all the three happen to contain $(NH_4)_2SO_4$ as nitrogen source. Low level of P solubilization was observed in other media containing NH_4NO_3 (Kim et al., 1997; Vassileva, 1998) and NBRIP medium containing less $(NH_4)_2SO_4$ as nitrogen sources.

A number of fungi had been reported of being able to solubilize phosphate only in the presence of ammonium as the nitrogen source (Illmer and Schinner, 1992; Lapeyrie, 1991). The nitrogen source in salt form seems to be important, as it was necessary for increased phosphate solubilization of rock phosphate (Asea, 1988). Previous reports on phosphorus solubilizing microorganisms (Lapeyrie, 1991; Carlile and Watkinson, 1994) have attributed the differences in phosphate solubilization (when ammonium and nitrate were used) to the use of different mechanisms for the generation of acidity in the culture. Our observation was also similar. However, no significant relationship could be established between the quantity of phosphate solubilized and drop in pH. We observed that when urea was added in medium as nitrogen source, the pH drop was from 7 to 5.23 only, but there was a significant phosphorus solubilization (Table 2). The overall results of the study indicate that acid production was not the only reason for phosphate release into the medium. This finding was in agreement with data obtained earlier reports (Abd Alla, 1994; Whitelaw, 2000).

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