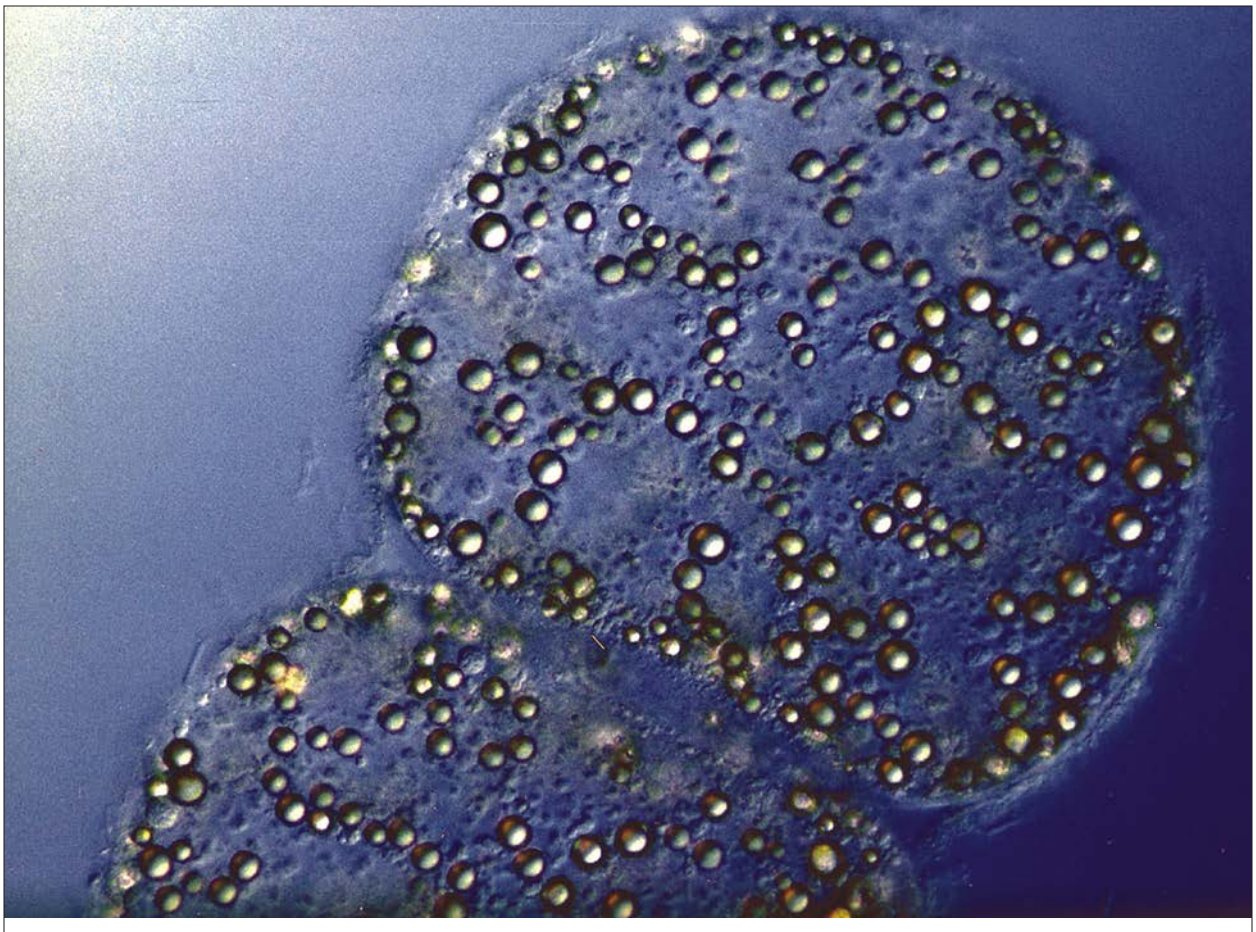

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Effect of Salt Concentration and pH on Soil Inhabiting Fungus *Penicillium citrinum* Thom. for Solubilization of Tricalcium Phosphate

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ABSTRACT

Aim of this studies, to improve phosphate solubilization activity of *Penicillium citrinum* at different salinity condition, carbon sources and varying level of pH. Plant Growth Promoting Fungus (PGPF) isolated from rhizosphere of sugarcane and identified as *Penicillium citrinum* Thom. was tested for its phosphate solubilizing ability on four carbon sources viz., glucose, sucrose, glycerol and mannitol, sources of salinity viz., NaCl, $CaCl_2$ and KCl at varying range of reaction (pH) *in vitro* condition. Among the carbon sources, *Penicillium citrinum* Thom. was found to be maximum significant solubilization of tricalcium phosphate (461 μg mL^{-1}) in presence of glucose as carbon source, while minimum phosphate solubilization (421 μg mL^{-1}) in presence of sucrose. Further, the effect of different salinity (NaCl, $CaCl_2$ and KCl) was tested at different pH (6.0, 7.0 and 8.0) under *in vitro* condition. Presence of 1% $CaCl_2$ at pH 8 in broth culture was maximum solubilization of tricalcium phosphate (455 μg mL^{-1}) than other salt concentration (NaCl and KCl) by this fungal strain of *P. citrinum* Thom. Results of this studies was found suitable carbon source as glucose for more solubilization of tricalcium phosphate. Also salinity effect on solubilization of tricalcium phosphate by *P. citrinum* was showed maximum significant in 1% $CaCl_2$ at pH 8.0. This finding can provide great benefit in the maintaining the available phosphates for crops in saline and alkaline soils. A large fraction of land arid and semiarid regions is affected by salinity in India. About 7.5 million of hectares of land are saline or alkaline, which is improved by *Penicillium citrinum*.

Key words: Rhizosphere, $CaCl_2$, NaCl, KCl, phosphate solubilization

INTRODUCTION

Plant growth promoting microorganisms have two major components that are Plant Growth Promoting Rhizobacteria (PGPR) and Plant Growth Promoting Fungi (PGPF). Both group of microorganism are equally important to enhance plant growth by means of mechanism of nutrition solubilization and their acquition to plants production of plant growth promoting substances and preventing the attack of pathogen. *Penicillium* sp. is also known as plant growth promoting fungi it has ability to solubilize fixed form of phosphorus and induced systematic resistance in plants

(Rodriguez *et al.*, 1996). Development of growth and activity of fungi is very much effected by source of carbon, nature and concentration of salt and pH of the soil (Johri *et al.*, 1999). Phosphorus is one of the major essential mineral fertilizers and is world's second largest agricultural chemical required by plant for its growth and development. Majority of the inorganic phosphorus applied to soil as a chemical fertilizer is rapidly fixed as insoluble forms (phosphates of iron, aluminum and calcium) and thus become unavailable to plants (Mittal *et al.*, 2008). In addition, chemical fertilizers are costly and have adverse effect on the soil fertility (Vassilev and Vassileva, 2003). Soils are often high in insoluble mineral and organic phosphates but deficient in available orthophosphate (Pi). Soil amendment with phosphatic fertilizer, produced via chemical processing of rock phosphate ore, is therefore an absolute requirement for crop reduction in order to feed the world's population. For over one hundred years, worker has recognized the ability of soil microorganism to solubilize Pi from insoluble (i.e., nutrionally unavailable) organic and mineral phosphates (Whitelaw, 2000). Wide range of microbial biosolubilization mechanism exists, so that much of global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi. To increase the availability of phosphorus for plants, large amount of fertilizer are used on a regular basis. But after application of large proportion of fertilizer phosphorus is quickly transferred to the insoluble form. Therefore very little percentage of the applied phosphorus is used making continuous application necessary. It has been reported that many soil inhibiting fungi and bacteria can solubilize inorganic phosphates.

Soil microbes have the ability to convert fixed form of phosphorus (in soil) to soluble forms that can be easily taken up by plants. High proportions of these Phosphate-Solubilizing Microorganisms (PSMs) are concentrated in the rhizosphere of plants (Vazquez *et al.*, 2000). Many studies have shown an increase in growth and P-uptake by plants through the inoculation of PSMs in pot experiments (Vassilev *et al.*, 2006) and under field conditions (Duponnois *et al.*, 2005; Valverde *et al.*, 2006).

Many bacterial, fungal, yeast and actinomycetes species capable of solubilizing sparingly soluble phosphorus in pure culture have been isolated and studied (Abd-Alla, 1994; Whitelaw, 2000). Application of PSMs in the field has been reported to increase crop yield. Species of *Aspergillus* and *Penicillium* are among fungal isolates identified to have phosphate solubilizing capabilities. The PSMs are a low-cost solution that enriches the soil giving a thrust to economic development without disturbing ecological balance. Several mechanisms like lowering the pH by acid production, iron chelating and exchange reaction in growth environment have been reported to play a vital role in phosphate solubilization by PSMs, fungi perform better in acidic soil conditions. Alkaline soils rich in calcium phosphate complexes have a very strong buffering capacity (Ae *et al.*, 1990). Yu *et al.* (2005), who investigated the solubilization of rock phosphate in liquid culture by *Aspergillus niger* and *Penicillium oxalicum*. Screening of phosphate solubilizing microbes using buffered media may lead to selection of more effective solubilizers (Gyaneshwar *et al.*, 1998). Many researchers have studied the effect of carbon sources of phosphate solubilization (Narsian and Patel, 2000). The aim of this study was to investigate levels of phosphates solubilization activity of *P. citrinum* Thom. under *in vitro* condition. The work forms part of our efforts towards understanding how to manage soil microbial communities based on specific functions (P solubilization) and selection of fungi as potential microbial inoculants (biofertilizers).

MATERIALS AND METHODS

Isolation and identification of fungus: Rhizospheric soil was collected from healthy sugarcane plant raised at Agricultural Research Farm, Institute of Agricultural Sciences, Banaras Hindu

University, Varanasi, (India) from 15-25 cm depth from the rhizoshere soil of Sugarcane (*Colletotrichum folcatum*) at January, 2008. The serial soil dilutions were spread plated on modified Pikovskaya's agar (Pikovaskya, 1948) containing 0.5% tricalcium phosphate (TCP) as the source of insoluble phosphate (Gupta *et al.*, 1994). The fungal colonies producing distinct zones of TCP solubilization were raised into pure cultures maintained on Potato Dextrose Agar (PDA) slants at 4°C and identify on the basis of cultural and microscopic features followed by the method of Subramanian (Subramanian, 1971; Barnett and Hunter, 1972). The phosphate solubilizing fungus identified as *Penicillium citrinum* Thom. at Agharkar Research Institute, Pune, Maharastra, India on the basis of phenotypic characters was selected for further studies on ability to solubilize TCP at different carbon source and effect of different salinity with different pH on the phosphate solubilizing activity of the isolated fungal strain, was also determined.

Media and growth condition: Phosphorus solubilizing ability of fungal strain was tested in five different carbon sources on modified PVK broth with 0.5% TCP. Effect of different carbon source on phosphate solubilization was done with addition of 1% respective sugars like glycerol, glucose, sucrose, mannitol. Further, we examined the phosphate solubilizing ability of fungus at different salts (NaCl, KCl and $CaCl_2$) at pH 6, 7 and 8 on modified Pikovskaya's broth with 0.5% TCP. Flasks were inoculated with 5% v/v, spore suspension and incubate on a orbital shaking incubator at 30°C for 7, 14 and 21 days.

Estimation of phosphorus: Cultures were harvested after different growth periods in order to record to change in pH and concentration of Phosphorus released in the medium. After centrifugation at 10,000 rpm for 30 min. The pH of 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 $\mu g\ mL^{-1}$ of phosphorus in culture medium.

Statistical analysis: The experiment was arranged in three replication. Statistical analysis was conducted using one-way analysis of variance (ANOVA). Comparisons of mean were performed by the Least Significant Difference (LSD) test at $p \leq 0.05$ by using SPSS software version 12.0.

RESULTS

Microorganisms: Fungi isolated from the different soils of agriculture research farm, Institute of agriculture sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India. Only this fungus *P. citrinum* Thom 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 tri-calcium phosphate, indicating phosphate solubilizing ability of the fungal isolate. It was selected for further studies. *Penicillium citrinum* Thom. showed greenish pigmentation with a rough surface.

Effect of different carbon sources and pH on *Penicillium citrinum* for Solubilization of insoluble phosphate: After confirming the phosphorus solubilizing ability on solid media, the phosphorus solubilization in liquid media was carried out in modified PVK broth using different

carbon source like sucrose, glycerol, mannitol, glucose at pH 7.0. For 7, 14 and 21 days incubation to find out suitable media formulation was more growth and solubilization of tricalcium phosphate for new isolated *P. citrinum* Thom. species. Modified PVK broth containing glucose as a carbon source showed maximum significant phosphorus solubilization at a rate of 324, 394 and 461 μg mL^{-1} phosphorus of cultured filtrate with resulting final pH of 5.9, 5.1and 4.8, respectively after incubation for 7, 14 and 21 days, respectively followed by glycerol as carbon source (Table 1). Low level of phosphorus solubilization was observed in modified PVK broth containing sucrose as a carbon source shows 295, 367 and 421 μg mL^{-1} phosphorus after 7, 14 and 21 days of incubation, respectively. There are drop in 7 pH if we used modified PVK broth 6.50, 5.90 and 5.70 after 7, 14 and 21 days of incubation, respectively (Table 1). Based on the above result we conclude that if glucose is used as a carbon source in PVK broth then phosphorus solubilization efficiencies have shown more by *P. citrinum*. So, glucose is a primary carbon source for our new isolates.

Effect of salinity on *Penicillium citrinum* solubilization of phosphorus: Phosphate solubilization activity of *P. citrinum* Thom. species was studied in the presence of three different salts, 1% NaCl, 1% $CaCl_2$ and 1% KCl in modified PVK broth at pH 6.0, 7.0 and 8.0. In presence of 1% NaCl, more solubilization was recorded in pH 7.0 after 21 days of inoculation (Table 2). In graph, according to increasing days of incubation was showed increased solubilization activity (Fig. 1).

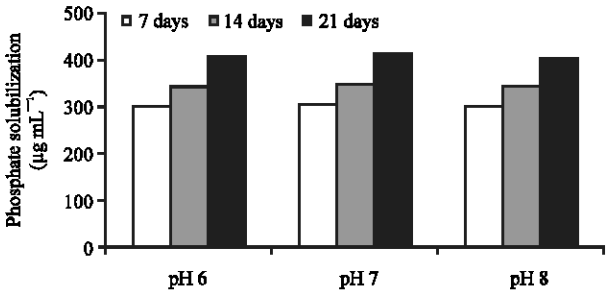

Fig. 1: Effect of 1% NaCl on phosphate solubilization activity of *Penicillium citrinum* Thom. species at different pH

Table 1: Effect of solubilization of Phosphorus by the fungus *Penicillium citrinum* Thom. in different carbon sources and noted media pH after 7, 14 and 21 days, respectively

	Week of incubation					
	7 days		14 days		21 days	
Carbon source	pH	PS*(μg mL^{-1})	pH	PS (μg mL^{-1})	pH	PS (μg mL^{-1})
Glucose	5.9	324^{b}	5.1	394^{b}	4.8	461^{c}
Sucrose	6.4	295^{a}	5.9	367^{a}	5.3	421^{a}
Maltose	6.3	310^{a}	5.9	378^{a}	5.1	438^{b}
Glycerol	6.1	321^{b}	5.7	388^{b}	5.0	451^{c}

PS: Phosphate solubilization; *Data are average values of three replicates; Mean with different letters in the same column differ significantly at $p \leq 0.05$ according to Fisher's protected LSD

Table 2: Effect of different pH and salt concentration on solubilization of tricalcium phosphate by *Penicillium citrinum*

	pH 6.0 (PS* in μg mL^{-1})			pH 7.0 (PS in μg mL^{-1})			pH 8.0 (PS in μg mL^{-1})		
Salt concentration	7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days
1% NaCl	229^{a}	314^{a}	407^{a}	305^{a}	347^{a}	414^{a}	301^{a}	14a^{3}	401^{a}
1% KCl	297^{b}	342^{b}	406^{a}	304^{a}	345^{a}	412^{a}	302^{a}	345^{b}	408^{a}
1% $CaCl_2$	319^{c}	369^{c}	439^{b}	328^{b}	378^{b}	448^{b}	340^{b}	390^{c}	455^{b}

PS: phosphate solubilization; *Data are average values of three replicates; Mean with different letters in the same column differ significantly at p≤0.05 according to Fisher's protected LSD

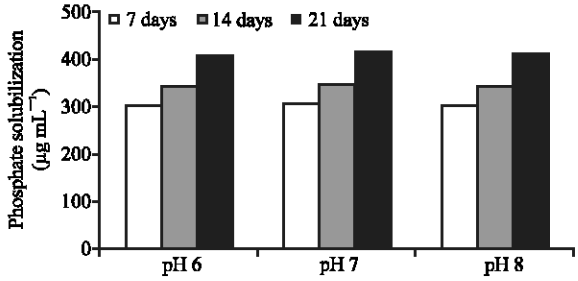

Fig. 2: Effect of 1% KCl on phosphate solubilization activity of *Penicillium citrinum* Thom. species at different pH

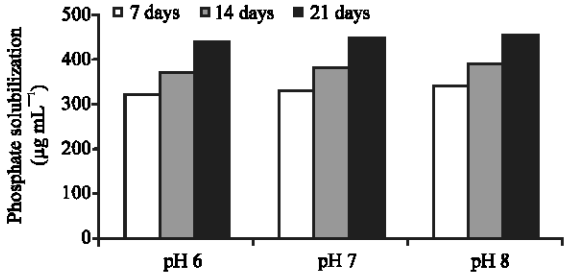

Fig. 3: Effect of 1% $CaCl_2$ on phosphate solubilization activity of *Penicillium citrinum* Thom. species at different pH

Similarly, in presence of 1% $CaCl_2$, more solubilization was recorded in pH 8.0 after 21 days of inoculation (Table 2). In Fig. 2 increasing activity of solubilization of tricalcium phosphate was increased according to period of incubation (Fig. 2). Production of acid was greatly effected by the nature of different salts. 1% $CaCl_2$ at pH 8 decrease the pH of the medium to the maximum extent and caused highest significant solubilization of phosphorus in respective to the 1% NaCl and 1% KCl. In presence of 1% KCl, more solubilization of tricalcium phosphate was showed at pH 7.0 after 21 days of incubation (Fig. 3). This strain demonstrated diverse level of phosphate solubilization activity in the presence of different salts at different pH. In control flask without any addition of salt growth did occur in the medium but drop in pH and phosphorus solubilization was quite low.

DISCUSSION

In the present study, the occurrence of phosphate solubilising organisms useful for tricalcium phosphate has been confirmed. Modified PVK broth containing glucose as a carbon source show maximum phosphorus solubilization phosphorus of cultured filtrate with resulting low pH. Glucose

is best carbon source for growth of *Penicillium citinum* and observed more significant solubilization of tricalcium phosphate. Similarly, Gaur (1990) reported that *Penicillium digitatum* solubilized the maximum P_2O_5 in the presence of glucose, followed by sucrose, mannitol, arabinose, fructose, xylose and galactose. Most of them could be used efficiently, but the minimum solubilization was observed in the presence of galactose. Rose (1957) showed that glucose or xylose was the best source of energy for fungi in liquid medium.

Phosphate solubilization activity of *P. citrinum* Thom. species was demonstrated in the presence of three different salts, 1 % NaCl, 1% $CaCl_2$ and 1% KCl in modified PVK broth at pH 6, 7 and 8. 1% $CaCl_2$ at pH 8 decrease the pH of the medium to the maximum extent and caused highest solubilization of phosphorus followed by 1% NaCl and 1% KCl. Acid production was increased due to addition of Ca compound in broth culture at pH 8.0. Similarly, Gluconic acid has been reported to be involved in the solubilization of Ca phosphate minerals by *Erwinia herbicola, Penicillium* sp. (Illmer and Schinner, 1992) and *Aspergillus niger* (Illmer and Schinner, 1995). Elnaghy and Megalla (1975) reported that the addition of Ca compound to *Pullularia pullulans* and to *Penicillium puberulum* culture solutions, respectively greatly increased the production of gluconic acid. Hydrogen ion concentration was affected the growth of microorganism. In present studied, the optimum pH (7.0 to 8.0) range for maximum tricalcium phosphate solubilization was recorded. Similarly results have been found by Pandey *et al.* (2008) were recorded between 320 μg mL^{-1} (*P. oxalicum*) and 500 μg mL^{-1} (*P. citrinum* and *P. purpurogenum*). In case of *P. oxalicum* the maximum solubilization (500 μg mL^{-1}) was attained on day 21of incubation due to more production of acid. The optimum pH 7.0 was recorded in presence of 1% NaCl and 1% KCl for solubilization of tricalcium phosphate. The optimum pH 8.0 was recorded in presence of 1% $CaCl_2$ for solubilization of tricalcium phosphate in broth culture. The optimum pH (5.0 to 7.6) range for maximum rock phosphate solubilization was studied by Gaur (1990). The maintained pH value changed appreciable due to sterilization of the medium. Such changes in pH value of the medium due to sterilization have been observed by several workers (Gaur, 1990). The selected efficient micro-fungi were capable of solubilizing rock phosphate over a wide range of pH from 5.0 to 7.8 (before sterilization pH value were 4.0 to 9.6). In control treatment without any addition of salt growth did occur in the medium but drop in pH and phosphorus solubilization was quite low. This is also confirmed by the fall of pH in culture filtrate, which was at maximum with TCP in fungi. Instead, in salt concentration in medium, pH drifted to the alkaline side. The order of solubilization of the phosphates of Ca>Al>Fe observed for these organisms is consistent with earlier reports for many phosphate solubilizing *Aspergillus* sp. (Vassileva *et al.*, 1998; Kang *et al.*, 2002; Gupta *et al.*, 2007; El-Azouni, 2008; Mittal *et al.*, 2008; Ogbo, 2010). There is however, a wide variation in the concentration of s oluble phosphates (9.47-1235 mg L^{-1}) reported to have been released during these studies. Lapeyrie *et al.* (1991) have suggested that P solubilization ability can be variable even within the same fungal species. The effect of pH on phosphate solubilization by fungi showed that pH 9 and 7.2 was suitable for solubilization of TCP in their presence. Fungi were naturally grown better under acidic than alkaline pH conditions because P solubilization is associated with production of acids, alkaline medium will tend to impair this process by neutralization of acidity. This can be seen from the corresponding increase in pH values recorded at the end of fermentation in PVK medium as initial pH was raised (Ogbo, 2010). Several reports have mentioned the effects of carbon and nitrogen sources on phosphate solubilization capacity and its enhancements. Similarly, nutritional modification and standardization of the C: N ratio can also enhance the phosphate solubilization activity of our microbial strain. The data of PSF on P-solubilizing

activity suggests that: *in vitro* values of P-solubilizing may be correlated with acid production levels under *in vivo* conditions (Mittal *et al*., 2008).

CONCLUSION

In present investigation, the phosphate solubilization ability was enhanced maximum in the presence of 1% $CaCl_2$ at pH 8 and glucose as carbon source by the strain *P. citrinum* Thom. It can provide great benefit in the maintaining the available phosphates for crops in saline and alkaline soils. A large fraction of land arid and semiarid regions is affected by salinity in India. About 7.5 million of hectares of land are saline or alkaline, which is improved by *Penicillium citrinum*.

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