



Trends in  
**Applied Sciences  
Research**

ISSN 1819-3579



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## Solubilization of Tricalcium Phosphate by Fungus *Aspergillus niger* at Different Carbon Source and Salinity

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### ABSTRACT

Plant Growth Promoting Fungus (PGPF) isolated from rhizosphere of chickpea and identified as *Aspergillus niger* strain BHUAS01 was tested for its tricalcium phosphate solubilizing ability at different sources of carbon viz., glucose, sucrose, glycerol and mannitol in Pikovskaya broth. Also, *A. niger* was analyzed solubilization of tricalcium phosphate (TCP) in broth media at different salinity viz., 1% NaCl, 1% KCl and 1% CaCl<sub>2</sub> at varying range of reaction (pH) under *in vitro* condition. Among the carbon sources, *Aspergillus niger* was found to solubilize maximum tricalcium phosphate (512 µg mL<sup>-1</sup>) at glucose as carbon source and minimum activity (348 µg mL<sup>-1</sup>) of phosphate solubilization at sucrose as carbon. *Aspergillus niger* showed maximum significant solubilization of tricalcium phosphate in Pikovskaya broth containing carbon source glucose followed by glycerol, maltose and sucrose at 21 days of incubation. Further the effect of different salinity (1% NaCl, 1% KCl and 1% CaCl<sub>2</sub>) was tested at different pH (6.0, 7.0 and 8.0) under *in vitro* condition. *A. niger* strain BHUAS01 was showed maximum significant solubilization of tricalcium phosphate (495 µg mL<sup>-1</sup>) in presence of 1% CaCl<sub>2</sub> in modified Pikovskaya broth at pH 8.0 than other salt concentration. 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.

**Key words:** PGPF, rhizosphere, *Aspergillus niger*, tricalcium phosphate, solubilization

### INTRODUCTION

Plant growth promoting microorganisms have two major components that are Plant Growth Promoting Rhizobacteria (PGPR) and Plant Growth Promoting Fungi (PGPF). 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. The optimal development of crops demands a high, often costly and input of P fertilizers. Current concepts in sustainability involve application of alternative strategies based on the use of less expensive natural sources of plant nutrients like rock phosphate. In addition, chemical fertilizers are costly and have adverse effect on the soil fertility (Vassilev and Vassileva, 2003). Soil microbes have the ability to convert fixed form of phosphorus (in soil) to soluble forms that can be easily taken up by plants (Rodriguez and Fraga, 1999). Phosphate-solubilizing microorganisms include different groups of microorganisms, which not only assimilate

phosphorus from insoluble forms of phosphates, but they also cause a large portion of soluble phosphates to be released in quantities in excess of their requirements. Species of *Aspergillus* and *Penicillium* are among fungal isolates identified to have phosphate solubilizing capabilities. Among the bacterial genera with this capability are *Pseudomonas*, *Azospirillum*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Arthrobacter*, *Alcaligenes*, *Serratia*, *Enterobacter*, *Acinetobacter*, *Flavobacterium* and *Erwinia* (Rodriguez *et al.*, 1996).

Seed or soil inoculation with Phosphate-Solubilizing Microorganisms (PSMs) is known to improve solubilization of fixed soil phosphorus and applied phosphates resulting in higher crop yields (Jones and Darrah, 1994). High proportions of these 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 (Omar, 1998; Valverde *et al.*, 2006) and under field conditions (Duponnois *et al.*, 2005; Valverde *et al.*, 2006).

Filamentous fungi are widely used as producers of organic acids (Mattey, 1992) and in particular *Aspergillus niger* and some *Penicillium* species have been tested in fermentation system or inoculated directly into soil in order to solubilize rock phosphate (Kucey, 1987). Reddy *et al.* (2002) found that all the isolates of *Aspergillus tubingensis* and *A. niger* isolated from rhizospheric soils were found to be capable of solubilizing all the natural forms of rock phosphates. This is the first report of solubilization of rock phosphates by *Aspergillus tubingensis* and showed that this fungus might serve as an excellent rock phosphate solubilizer when inoculated into soils where rock phosphate is used as P fertilizer. Goenadi *et al.* (2000) determined the optimum incubation period and the optimum level of rock phosphate for a Phosphate Solubilizing Fungus (PSF), *Aspergillus niger* BCCF.194, isolated from tropical acid soils. 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). 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 (Halder *et al.*, 1991; Narison and Patel, 2000). Therefore, the present investigation was under taken to find suitable carbon source, salt and pH for the solubilization of unavailable form of phosphorus in vitro condition by the fungus *Aspergillus niger* strain BHUAS01.

## **MATERIALS AND METHODS**

**Isolation and identification of fungus:** Rhizospheric soil was collected from healthy chickpea (*Cicer arietinum* L.) plant raised at Agricultural Research Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, (India) from 15-25 cm depth at Year 2007. Ten gram of soil was taken and made serial dilution in sterilized distilled water. The serial soil dilutions were spread plated on Pikovskaya's (PKV) agar containing 0.5% tricalcium phosphate (TCP) as the source of insoluble phosphate (Gupta *et al.*, 1994). Ingredient (g L<sup>-1</sup>) of Pikovskaya's agar (Pikovskaya, 1948) is Yeast extract, 0.50; Dextrose, 10.00; Calcium phosphate, 5.00; Ammonium sulfate, 0.50; Potassium chloride, 0.20; Magnesium sulfate, 0.10 Manganese sulfate, 0.0001; Ferrous sulfate, 0.0001; Agar, 15.00. The fungal colonies were produced halo zones on modified PKV agar plate at five days of incubation at 28±2°C. This colony of fungus was isolated as pure cultures and maintained on Potato Dextrose Agar slants at 4°C. This fungus culture was showed black color spore on PVK agar plate. This fungus was identified on the basis of cultural and microscopic

features followed by the method of Subramanian (Subramanian, 1971; Barnett and Hunter, 1972; Aneja, 2003). The phosphate solubilizing fungus 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 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<sub>2</sub>) at pH 6.0, 7.0 and 8.0 on modified Pikovskaya's broth with 0.5% TCP. Flasks were inoculated with 5% v/v, spore suspension and incubate on an orbital shaking incubator (Remi made) at 28±2°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 8000 rpm for 15 min. The pH of culture medium was measured with a pH meter equipped with a glass electrode. Dissolved phosphorus concentration in the culture filtrate was determined by Vanado-molybdate method as described by APHA (1995). It was expressed in terms of µg mL<sup>-1</sup> of phosphorus in culture medium.

## RESULTS

**Microorganisms:** Fungi isolates with the ability to solubilize insoluble phosphorus were isolated from soil characterized with high level of tricalcium phosphate. The fungal isolate that displayed the highest ratio of halo zone/colony diameter were selected and identified as *A. niger* at 5 days of incubation at 28±2°C. Fungus *A. niger* strain BHUAS01 showed significant zone of phosphate solubilization. *A. niger* strain BHUAC01 shows white mycelium with black spore.

**Solubilization of tricalcium phosphate:** The phosphorus solubilization in liquid media was carried out in PVK broth using different carbon source like glucose, sucrose, glycerol and mannitol at neutral pH for 7, 14 and 21 days incubation at 30°C to find out suitable media formulation for proper growth of *Aspergillus niger*. PVK broth containing glucose as a carbon source showed maximum significant tricalcium phosphate solubilization at the rate of 438, 453 and 512 µg mL<sup>-1</sup> phosphorus in broth culture with resulting final pH of 4.9, 4.0 and 3.5, respectively after incubation for 7, 14 and 21 days respectively. Less phosphorus solubilization was observed in PVK broth containing sucrose as a carbon source shows 348, 385 and 421 µg mL<sup>-1</sup> phosphorus after 7, 14 and 21 days of incubation, respectively (Table 1). Glucose is used as a sole source of carbon for growth of *Aspergillus niger* in PVK broth to enhanced solubilization of tricalcium phosphate as compare to other carbon source. So, glucose is a primary carbon source for *Aspergillus niger* strain BHUAS01 isolates.

**Effect of different salt on solubilization of phosphorus:** Phosphate solubilization activity of *A. niger* was observed in the presence of three different salts, 1% NaCl, 1% CaCl<sub>2</sub> and 1% KCl in PVK broth at pH 6.0, 7.0 and 8.0 (Table 2). This strain demonstrated diverse level of phosphate solubilization activity in the presence of different salts at different pH. Production of various organic acids by *Aspergillus niger* was greatly effected by the nature of different salts.

Table 1: Effect of solubilization of phosphorus by the fungus *Aspergillus niger* BHUAS01 in different carbon sources and noted media pH after 7, 14 and 21 days, respectively

| Carbon source | Week of incubation |                              |         |                              |         |                              |
|---------------|--------------------|------------------------------|---------|------------------------------|---------|------------------------------|
|               | 7 days             |                              | 14 days |                              | 21 days |                              |
|               | pH                 | PS ( $\mu\text{g mL}^{-1}$ ) | pH      | PS ( $\mu\text{g mL}^{-1}$ ) | pH      | PS ( $\mu\text{g mL}^{-1}$ ) |
| Glucose       | 4.9                | 438 <sup>c</sup>             | 4.0     | 453 <sup>c</sup>             | 3.5     | 512 <sup>d</sup>             |
| Sucrose       | 5.7                | 348 <sup>a</sup>             | 5.1     | 385 <sup>a</sup>             | 4.4     | 421 <sup>a</sup>             |
| Maltose       | 5.2                | 360 <sup>a</sup>             | 4.7     | 395 <sup>a</sup>             | 4.1     | 438 <sup>b</sup>             |
| Glycerol      | 5.8                | 385 <sup>b</sup>             | 4.3     | 425 <sup>b</sup>             | 3.8     | 461 <sup>c</sup>             |

<sup>a</sup>Data are average values of three replicates. Mean with different letter(s) in the same column differ significantly at  $p = 0.05$  according to Fisher's Protected LSD. PS: Phosphate solubilization

Table 2: Effect of different pH and salt concentration on solubilization of tricalcium phosphate ( $\mu\text{g mL}^{-1}$ ) by *Aspergillus niger* BHUAS01

| Salt concentration   | pH 6.0           |                  |                  | pH 7.0           |                  |                  | pH 8.0           |                  |                  |
|----------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|                      | 7 days           | 14 days          | 21 days          | 7 days           | 14 days          | 21 days          | 7 days           | 14 days          | 21 days          |
| 1% NaCl              | 316 <sup>a</sup> | 344 <sup>a</sup> | 417 <sup>a</sup> | 315 <sup>a</sup> | 367 <sup>a</sup> | 395 <sup>a</sup> | 311 <sup>a</sup> | 374 <sup>a</sup> | 413 <sup>a</sup> |
| 1% KCl               | 318 <sup>a</sup> | 342 <sup>a</sup> | 426 <sup>a</sup> | 326 <sup>a</sup> | 385 <sup>a</sup> | 418 <sup>a</sup> | 322 <sup>a</sup> | 385 <sup>b</sup> | 428 <sup>b</sup> |
| 1% CaCl <sub>2</sub> | 336 <sup>c</sup> | 369 <sup>c</sup> | 449 <sup>b</sup> | 358 <sup>b</sup> | 398 <sup>b</sup> | 478 <sup>b</sup> | 346 <sup>b</sup> | 396 <sup>b</sup> | 495 <sup>c</sup> |

<sup>a</sup>Data are average values of three replicates. Mean with different letter(s) in the same column differ significantly at  $p = 0.05$  according to Fisher's Protected LSD

*Aspergillus niger* strain BHUAS01 showed statistically higher significant solubilization of tricalcium phosphate at 1% CaCl<sub>2</sub> in PVK broth as compare to 1% NaCl and 1% KCl at three different pH 6.0, 7.0 and 8.0 (Table 2). Production of acid was greatly effected by the nature of different salts. One percent CaCl<sub>2</sub> at pH 8.0 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 (Table 2). 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 solubilizing organisms useful for tricalcium phosphate has been confirmed. *Aspergillus niger* showed halo zone around their colony on PVK agar plate. The appearance of a clear halo zone around the colony indicated phosphate solubilization by the fungus (Kang *et al.*, 2002; Gupta *et al.*, 2007). The advantage of using natural phosphate solubilizers over the genetically manipulated or ones that have been isolated from a different environmental sets-up is the easier adaptation and succession when inoculated into the medium containing RP (Xiao *et al.*, 2008). PVK broth containing glucose as a carbon source showed maximum phosphorus solubilization of phosphorus in broth culture with resulting low pH.

Glucose is best carbon source for growth of *Aspergillus niger* strain BHUAS01 and observed more significant solubilization of tricalcium phosphate. Similarly, Gaur (1990) reported that *Penicillium digitatum* solubilized the maximum P<sub>2</sub>O<sub>5</sub> in the presence of glucose, followed by

sucrose, mannitol, arabinose, fructose, xylose and galactose. Other researchers, Yadav *et al.* (2010) reported that *Penicillium citrinum* was solubilized tricalcium phosphate in presence of glucose as sole source of carbon, followed by glycerol, maltose and sucrose. Similar result found by Saber *et al.* (2009) the glucose was the best source of carbon for growth of *Aspergillus niger* and *Penicillium* sp. in broth medium.

Phosphate solubilization activity of *Aspergillus niger* strain BHUAS01 was demonstrated in the presence of three different salts, 1% NaCl, 1% CaCl<sub>2</sub> and 1% KCl in modified PVK broth at pH 6.0, 7.0 and 8.0. *Aspergillus niger* was showed statistical more significant tricalcium phosphate solubilization at 1% CaCl<sub>2</sub> followed by 1% NaCl and 1% KCl at pH 8.0. 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* (Liu *et al.*, 1992), *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. Narison and Patel (2000) studied the influence of chelators on phosphate solubilization by *Aspergillus aculeatus*, a rhizosphere isolate of gram. They concluded that different test chelators had differential behaviors in relation to phosphate solubilization. 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) was recorded between 320 µg mL<sup>-1</sup> (*P. oxalicum*) and 500 µg mL<sup>-1</sup> (*P. citrinum* and *P. purpurogenum*). In case of *P. oxalicum* the maximum solubilization (500 µg mL<sup>-1</sup>) was attained on 21 days of 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<sub>2</sub> 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 soluble phosphates (9.47-1235 mg L<sup>-1</sup>) 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).

## CONCLUSION

In present study, *Aspergillus niger* strain BHUAS01 showed maximum significant solubilization of tricalcium phosphate at 1% CaCl<sub>2</sub> in saline condition and glucose used as sole source of carbon for more growth of fungus. It can provide great benefit in the maintaining the available phosphates for crops in saline and alkaline soils.

## ACKNOWLEDGMENT

The authors are thankful to Department of Soil Science and Agricultural Chemistry for providing necessary facility for completing this research work.

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