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# Strain Improvement of Phosphate Solubilizing Fungal Strains

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***Abstract***

A laboratory study was conducted to isolate, investigate and enhance the phosphate solubilization efficiency of certain isolated fungal strains viz., *Aspergillus niger, Aspergillus fumigatus, Penicillium sp*. These fungal strains were isolated from the rhizosphere of paddy fields in Tamilnadu, India and screened for phosphate solubilization. The enhancement of phosphate solubilization of these strains was studied through three types of mutagenesis: UV, sodium azide and ethyl methane sulphonate mutagenesis. The mutant ANuv60 exhibited 127% increased efficiency for phosphate solubilization compared to the wild strains. Similarly, the phosphate solubilization by sodium azide mutant (ANsa120) was 133% higher and EMS mutant (ANems120) was 135% higher than the wild strain. The efficient strains were further studied for the effect of carbon and nitrogen source on phosphate solubilization. Significance of the findings is discussed in detail.

***Abbreviation***

SA: Sodium azide, EMS: Ethyl methane sulphonate, UV: Ultra Violet, ANsa: Sodium azide treated *Aspergillus niger,* AFsa: Sodium azide treated *Aspergillus fumigatus,* PEsa: Sodium azide treated *Penicillium.* ANems: Ethyl methane sulphonate treated *Aspergillus niger,* AFems: Ethyl methane sulphonate treated *Aspergillus fumigatus,* PEems: Ethyl methane sulphonate treated *Penicillium,* ANuv: Ultra Violet treated *Aspergillus niger,* AFuv: Ultra Violet treated *Aspergillus fumigatus* and PEuv: Ultra Violet treated *Penicillium.*

## 1. Introduction

Phosphorus is called "the key of life" as it is directly involved in most life processes. Next to nitrogen it is invariably classified as one at the macronutrients and is an important key element in frequency of use as fertilizer. It serves as a primary energy source for microbial oxidation. It is a constituent substance in life processes. Soil can not give high yields if it is deficient in phosphate. Soil contains both organic and inorganic forms of phosphorus. The organic forms of the element in soil are compounds of phytins, phospholipids and nucleic acids that come mainly by the way of decaying vegetation.

Plants take phosphate in the form of soluble orthophosphate ions but due to the presence of Ca, Mg, K, Na, Al and Fe ions in soil, the soluble orthophosphate is converted to insoluble form. Because of this process plants utilize very little amount of phosphate, even though phosphatic fertilizers are added (Vivek kumar *et al.,* 1999; Rosas *et al*., 2006; Jayasinghearachchi and Seneviratne, 2006). Soil microorganisms are reported to be initially associated in the cycling of phosphorus. But they also participate in solubilization of inorganic phosphorus and in the mineralization of organic phosphorus (Agnihothri, 1970; Ostwal and Bhide, 1972).

A large fraction of microbial population can dissolve insoluble inorganic phosphorus known to occur in the soil (Hayman, 1975). Various microorganisms were reported to solubilize different types of insoluble phosphates. Bacteria such as *Achromobacter* sp, *Agrobacterium* sp, Fungi like *Aspergillus niger, A. flavus, A. fumigatious, Penicillium* sp, *Rhizopus* sp etc are the important phosphate solubilizers present in the soil.

Different mechanisms have been suggested for the solubilization of inorganic phosphorus by phosphate solubilizers. The production of organic acids in the microenvironment around the root or in the culture media is considered to be the important cause of phosphate solubilization (Sperber, 1957; Bardiya and Gaur, 1972). Achal *et al.* (2007) reported the phenotypic mutants of *Aspergillus tubingensis* were obtained by UV irradiation and phosphate solubilization ability of these mutants were studied and compared with wild type strains. However, not much work has been carried on phosphate solubilization by mutated *Aspergillus* and *Penicillium* species. Hence the present study has been undertaken to study reveals the efficacy of phosphate solubilization by mutated strains isolated from paddy soils.

## 2. Materials and Methods

### Microorganisms and isolation

The fungal strains were isolated from rhizosphere soils of Paddy field, Mannachanallur,

one of the largest paddy producing taluk located in Tamil Nadu State, India. The soil samples were screened for phosphate solubilizing fungi in Pikovskaya's agar medium by conventional dilution plate count method. The fungal strains showing halo formation around their colonies were isolated and grown on Sabouraud agar slants at 27°C for 3 days and kept in the refrigerator at $4^0$C until further use (Ellaiah *et al*., 2002).

**Identification**

The fungal cultures were identified based on the colony morphology and spore structure (Pradhan and Sukla, 2005)

**Random mutational studies**

**Mutagenesis by UV**

72 hrs old fungal cultures were scraped off from agar slants and suspended in 5ml sterile distilled water and then diluted with 45 ml of sterile distilled water containing Tween 80 (1:4000). Sterile glass beads were added to the suspension and kept on rotary shaker for 30 min to break the hyphal mycelium. The suspension was filtered to remove the mycelium. The spore suspension was prepared in phosphate buffer (pH7.0) containing $10^6$ spores per ml. Five ml quantities of the spore suspension were transferred aseptically into sterile petri dishes and exposed to UV light (2600 $A^o$) at a distance of 15cm away from the center of the Germicidal lamp for various time intervals (10, 20, 30, 40, 50, 60, 70 and 80 min). The suspension was agitated by gently rotating the plates in between the time intervals. (Ellaiah *et al*., 2002).

The UV exposed spore suspensions were stored in dark for overnight to avoid photo reactivation. After overnight incubation, irradiated spore suspensions were serially diluted by using phosphate buffer (pH 7.0) and plated on Sabouraud's Dextrose agar medium. The plates were incubated for 5 days at 27°C. The colonies were selected on the basis of their morphological characters and were given the code numbers ANuv30, ANuv40, ANuv50, ANuv60, AFuv30, AFuv40, AFuv50, AFuv60, PEuv30, PEuv40, PEuv50 and PEuv60.

**Mutagenesis by Chemical**

Chemical mutagenesis was performed using Sodium azide and Ethyl methane sulphonate (EMS) for the strain improvement of phosphate solubilizers. Spore suspensions of fungal strains were prepared by using phosphate buffer pH 7.0 as described earlier (Ellaiah *et al*., 2002). To 9 ml of each spore suspension, 1 ml of sterile solution of Sodium azide (250 $\mu g\ ml^{-1}$ in phosphate buffer) was added. Similar procedure was adopted for Ethyl methane sulphonate (EMS) (150 $\mu g\ ml^{-1}$ in phosphate buffer). The reaction was allowed to proceed. Control tube was also kept without any chemical mutagen. Samples were withdrawn from the reaction mixture at an interval of 30, 60, 90, 120 and 150 min. and centrifuged for 10 min. at 5000 rpm. The cells were washed three times with sterile distilled water and again re-suspended in 10 ml sterile buffer. The samples were serially diluted in the same buffer and plated on Sabouraud's Dextrose agar medium. The selected sodium azide treated mutants were given the code numbers ANsa30, ANsa60, ANsa90, ANsa120, AFsa30, AFsa60, AFsa90, AFsa120, PEsa60, PEsa90, PEsa120 and PEsa150.

The EMS treated mutants were given the code numbers ANems30, ANems60, ANems90, ANems120,AFems30, AFems60, AFems90, AFems120,PEems60, PEems90, PEems120 and PEems150 (Bapiraju *et al.,* 2004).

**Submerged fermentation**

**Inoculation**

Pure fungal cultures from agar slants were inoculated to Sabouraud's Dextrose agar plates and incubated for four days at 27°C. After sufficient growth, 8 mm discs (~$10^6$ spores/ml) were cut from the plates using sterile cork borer and were inoculated into 100 ml Pikovskaya's broth (Narsian *et al.,* 1995). The flasks were incubated at 27°C for 9days. Control Pikovskaya's broth medium was also kept and the experiment was conducted in triplicate set.

**Phosphate estimation**

Broth culture medium was withdrawn aseptically at three days interval from each flask and centrifuged at 10,000 rpm for 20 minutes (Dave and Patel, 1999). The supernatant was estimated for phosphate content by chlorostannous reduced molybdophosphoric blue colour method (Seshadri, 1995).

**Effect of different carbon sources**

Effect of different carbon sources on phosphate solubilization efficiency of the isolated strains was also studied. The carbon source used was maltose, sucrose and mannitol instead of glucose. Efficient phosphate solubilizing fungal strains were inoculated in to broth media with various carbon sources and incubated. After incubation, phosphate solubilization assay was carried out (Narsian and Patel, 2000).

**Effect of different nitrogen sources**

To study the effect of different nitrogen sources on phosphate solubilization efficiency of the fungal strains, the nitrogen source ammonium sulphate was replaced by potassium nitrate, sodium nitrate and urea. Efficient phosphate solubilizing

fungal strains were inoculated and incubated. After incubation, phosphate solubilization assay was carried out (Narsian and Patel, 2000).

## 3. Results and Discussion

The efficacy of phosphate solubilization of fungal strains treated by ultraviolet rays, sodium azide and ethyl methane sulphonate (EMS) are presented in the Tables 1, 2 and 3 respectively. The influence of different carbon and nitrogen sources on phosphate solubilization of fungal mutants are reported in the Tables 4 and 5.

Table 1. Efficacy of phosphate solubilization of fungal strains treated by UV

| UV irradiated *Aspergillus niger* strains | *$^{3}$P ppm of filtrate (Days) | | | Increased % of phosphate Solublization (Days) | | | UV irradiated *Aspergillus fumigatus* strains | *$^{3}$P ppm of filtrate (Days) | | | Increased % of phosphate solublization (Days) | | | UV irradiated *Penicillium sp* | *$^{3}$P ppm of filtrate (Days) | | | Increased % of phosphate solublization (Days) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3rd | 6th | 9th | 3rd (%) | 5th (%) | 9th (%) | | 3rd | 6th | 9th | 3rd (%) | 5th (%) | 9th (%) | | 3rd | 6th | 9th | 3rd (%) | 5th (%) | 9th (%) |
| ANuv30 | 2.65 | 4.05 | 5.00 | 20.45 | 10.96 | 8.70 | AFuv30 | 3.25 | 3.65 | 3.85 | 10.17 | 12.31 | 5.48 | PEuv30 | 3.45 | 4.10 | 4.25 | 4.55 | 6.49 | 6.25 |
| ANuv40 | 2.50 | 3.85 | 4.75 | 13.64 | 5.48 | 3.26 | AFuv40 | 3.15 | 3.65 | 4.15 | 6.78 | 12.31 | 13.70 | PEuv40 | 3.65 | 4.25 | 4.45 | 10.61 | 10.39 | 11.25 |
| ANuv50 | 2.85 | 4.35 | 5.50 | 29.55 | 19.18 | 19.57 | AFuv50 | 3.55 | 3.95 | 4.55 | 20.34 | 21.54 | 24.66 | PEuv50 | 3.75 | 4.40 | 4.75 | 13.64 | 14.29 | 18.75 |
| ANuv60 | 3.05 | 4.65 | 5.85 | 38.64 | 27.40 | 27.17 | AFuv60 | 3.35 | 3.85 | 4.45 | 13.56 | 18.46 | 21.92 | PEuv60 | 3.40 | 4.20 | 4.35 | 3.03 | 9.09 | 8.75 |
| Wild type[a] | 2.20 | 3.65 | 4.60 | | | | Wild type[a] | 2.95 | 3.25 | 3.65 | | | | Wild type[a] | 3.30 | 3.85 | 4.00 | | | |

* Values are expressed as mean ± standard deviation (>0.09) of triplicates
[a] Standard for calculation of increased %

Table2. Efficacy of phosphate solubilization of fungal strains treated by sodium azide

| Sodium azide treated *Aspergillus niger* strains | *$^{3}$P ppm of filtrate (Days) | | | Increased % of phosphate Solublization (Days) | | | Sodium azide treated *Aspergillus fumigatus* strains | *$^{3}$P ppm of filtrate (Days) | | | Increased % of phosphate solublization (Days) | | | Sodium azide treated *Penicillium sp* | *$^{3}$P ppm of filtrate (Days) | | | Increased % of phosphate solublization (Days) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3rd | 6th | 9th | 3rd (%) | 5th (%) | 9th (%) | | 3rd | 6th | 9th | 3rd (%) | 5th (%) | 9th (%) | | 3rd | 6th | 9th | 3rd (%) | 5th (%) | 9th (%) |
| ANsa30 | 2.40 | 4.10 | 5.05 | 9.0 | 12.3 | 9.7 | AFsa30 | 3.10 | 3.45 | 3.95 | 5.0 | 6.1 | 8.2 | PEsa60 | 3.50 | 4.15 | 4.35 | 6.0 | 7.7 | 8.7 |
| ANsa60 | 2.50 | 4.30 | 5.55 | 13.6 | 17.8 | 20.6 | AFsa60 | 3.25 | 3.75 | 4.25 | 10.1 | 15.3 | 16.4 | PEsa90 | 3.60 | 4.50 | 5.05 | 9.0 | 16.8 | 26.2 |
| ANsa90 | 2.95 | 4.65 | 5.80 | 34.0 | 27.4 | 26.0 | AFsa90 | 3.45 | 4.00 | 5.30 | 16.9 | 23.0 | 45.0 | PEsa120 | 3.70 | 4.65 | 5.65 | 12.1 | 20.7 | 41.2 |
| ANsa120 | 3.45 | 4.95 | 6.15 | 56.8 | 35.6 | 33.6 | AFsa120 | 4.05 | 4.65 | 5.50 | 37.0 | 43.0 | 50.6 | PEsa150 | 3.80 | 4.85 | 5.95 | 15.1 | 25.9 | 48.7 |
| Wild type[a] | 2.20 | 3.65 | 4.60 | | | | Wild type[a] | 2.95 | 3.25 | 3.65 | | | | Wild type[a] | 3.30 | 3.85 | 4.00 | | | |

*Values are expressed as mean ± standard deviation (>0.09) of triplicates
[a] Standard for calculation of increased %

Table 3. Efficacy of phosphate solubilization of fungal strains treated by Ethyl Methyl Sulphonate (EMS)

| EMS treated *Aspergillus niger* strains | *$^{3}$P ppm of filtrate (Days) | | | Increased % of phosphate Solublization (Days) | | | EMS treated *Aspergillus fumigatus* strains | *$^{3}$P ppm of filtrate (Days) | | | Increased % of phosphate solublization (Days) | | | EMS treated *Penicillium sp* | *$^{3}$P ppm of filtrate (Days) | | | Increased % of phosphate solublization (Days) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3rd | 6th | 9th | 3rd (%) | 5th (%) | 9th (%) | | 3rd | 6th | 9th | 3rd (%) | 5th (%) | 9th (%) | | 3rd | 6th | 9th | 3rd (%) | 5th (%) | 9th (%) |
| ANems30 | 2.30 | 3.90 | 4.75 | 4.5 | 6.8 | 3.2 | AFems30 | 3.25 | 3.45 | 3.85 | 10.1 | 6.1 | 5.4 | PEems60 | 3.65 | 4.70 | 5.15 | 10.6 | 22.0 | 28.7 |
| ANems60 | 2.50 | 4.25 | 5.40 | 13.6 | 16.4 | 17.3 | AFems60 | 3.55 | 3.80 | 4.15 | 20.3 | 16.9 | 13.6 | PEems90 | 4.05 | 4.90 | 5.30 | 22.7 | 27.2 | 32.5 |
| ANems90 | 2.65 | 4.45 | 5.65 | 20.4 | 21.9 | 22.8 | AFems90 | 3.75 | 4.15 | 4.65 | 27.1 | 27.6 | 27.3 | PEems120 | 4.55 | 5.10 | 5.45 | 37.8 | 32.4 | 36.2 |
| ANems120 | 2.85 | 4.75 | 6.25 | 29.5 | 30.1 | 35.8 | AFems120 | 3.95 | 4.45 | 5.15 | 33.8 | 36.9 | 41.0 | PEems150 | 4.80 | 5.70 | 5.95 | 45.4 | 48.0 | 48.7 |
| Wild type[a] | 2.20 | 3.65 | 4.60 | | | | Wild type[a] | 2.95 | 3.25 | 3.65 | | | | Wild type[a] | 3.30 | 3.85 | 4.00 | | | |

* Values are expressed as mean ± standard deviation (>0.09) of triplicates
[a] Standard for calculation of increased %

Table 4. Efficacy of phosphate solubilization of fungal strains grown on different carbon sources

| Carbon sources vs ANems120 strains | *'P' ppm of filtrate (Days) 3rd | 6th | 9th | Carbon sources vs ANsa120 Strains | *'P' ppm of filtrate (Days) 3rd | 6th | 9th | Carbon sources PEsa150 | *'P' ppm of filtrate (Days) 3rd | 6th | 9th |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Glucose | 2.85 | 4.30 | 6.25 | Glucose | 3.45 | 4.95 | 6.15 | Glucose | 3.80 | 4.25 | 5.95 |
| Sucrose | 2.40 | 3.75 | 4.85 | Sucrose | 3.55 | 4.55 | 4.95 | Sucrose | 3.35 | 3.90 | 4.60 |
| Mannitol | 2.05 | 3.05 | 4.35 | Mannitol | 1.85 | 2.15 | 2.90 | Mannitol | 3.05 | 3.75 | 4.25 |
| Lactose | 2.20 | 3.60 | 4.60 | Lactose | 3.05 | 3.55 | 3.95 | Lactose | 3.15 | 3.80 | 4.35 |

*Values are expressed as mean ± standard deviation (>0.09) of triplicates

Table 5. Efficacy of phosphate solubilization of fungal strains grown on different nitrogen sources

| Nitrogen sources vs ANems120 strains | *'P' ppm of filtrate (Days) 3rd | 6th | 9th | Nitrogen sources vs ANsa120 Strains | *'P' ppm of filtrate (Days) 3rd | 6th | 9th | Nitrogen sources PEsa150 | *'P' ppm of filtrate (Days) 3rd | 6th | 9th |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Amm.sul. | 2.85 | 4.30 | 6.25 | Amm. Sul. | 3.45 | 4.95 | 6.15 | Amm. Sul. | 3.80 | 4.25 | 5.95 |
| Sod. Nit. | 1.90 | 2.65 | 3.20 | Sod. Nit. | 1.75 | 2.30 | 2.75 | Sod. Nit. | 3.30 | 3.75 | 4.05 |
| Pot. Nit. | 2.35 | 3.70 | 4.70 | Pot. Nit. | 2.40 | 3.70 | 4.65 | Pot. Nit. | 3.35 | 3.90 | 4.60 |
| Urea | 2.40 | 3.70 | 4.75 | Urea | 2.45 | 3.85 | 4.70 | Urea | 3.70 | 3.95 | 4.70 |

* Values are expressed as mean ± standard deviation (>0.09) of triplicates

Phosphate solubilization efficacy of the wild strains was observed in the descending order of *Aspergillus niger* >.*Penicillium Sp* > *Aspergillus fumigatus*. In the first part of the study, among the UV irradiated fungal strains, ANuv60 was found as the predominant phosphate solubilizing strain followed by ANuv50. In the second part of the study involving chemical mutagenesis of phosphate solubilizing strains, ANems120 showed the highest phosphate solubilization followed by ANsa120. Achal *et al.* (2007) reported a significant increase in soluble phosphate level was observed in case of UV induced mutants of *Aspergillus* strains compared with wild strains. The present study also revealed that UV induced mutant enhanced the phosphate solubilization compared with wild strains. There might be a possibility of alteration at genetic levels in case of mutants. Tripura *et al.* (2007) reported that the EMS treated microbial strains have increased phosphate solubilization efficiency compared to the wild strains.

The phosphate solubilization efficacy of mutant fungal strains (ANems120, ANsa120 and PEsa150) were demonstrated in the presence of various carbon and nitrogen sources. The effect of different carbon sources on phosphate solubilization efficiency of fungal strains was found to be in the ascending order mannitol < lactose < sucrose < glucose. Seshadri *et al.* (2004) reported that the mannitol was the best carbon source utilized by fungi preferred for higher phosphate solubilization and the nitrogen in the form nitrate was very effective and urea was the poorest source of nitrogen by *Aspergillus niger*. The present study revealed that mannitol was the poorest carbon source and the urea was one of the predominant nitrogen sources for phosphate solubilization by *Aspergillus niger*. This may due to the genetic alteration in the fungus due to chemical mutation. Reyes *et al.* (1999) studied the carbon source sucrose appeared to be the best carbon source for phosphate solubilization by UV induced fungal strain. Nautiyal *et al.* (2000) reported that the glucose and lactose were the best carbon source and sucrose, sorbitol were identified as poor carbon source for phosphate solubilization, ammonium and nitrate source to be equally effective for phosphate solubilization. This is in contrary with the report of Halder *et al* (1991) and Abualla (1994).

Similarly, the addition of various nitrogen sources in the medium to determine their effect on phosphate solubilization efficiency of the inoculated strains gave the following results in the ascending order sodium nitrate < potassium nitrate < urea < ammonium sulphate. Vassileve *et al.* (1998) reported the encapsulated spores of *Aspergillus niger* solubilized rock phosphate in the culture medium due to the production of organic acids. Whitelaw *et*

*al.* (1999) reported that the *Penicillium* strain solubilized the insoluble phosphate in the culture medium containing ammonium or nitrate as sole source of nitrogen. Vassileve *et al.* (2007) compared the phosphate solubilization efficacy of *Aspergillus niger* by using corn steep liquor, ammonium sulphate and yeast extract as nitrogen source.

The present study also indicates, the solubilization efficacy of the fungal strain *Aspergillus niger* treated with sodium azide (ANsa120) and *Penicillium* sp treated with EMS (PEems150) were about 1.5-fold higher when compared to wild strain. The chemical mutagenic agents such as sodium azide, ethyl methane sulphonate may alter the gene sequence by altering the base pairs. The azide ion alters the structure of cytosine such that it forms hydrogen bonds with adenine, rather than guanine. This produces a cytosine to thymine transition. Ethyl methane sulphonate is a strong mutagenic agent. It alkylates N7 of Guanine and severely alters the base pairing.

## 4. Conclusion

Many soil microorganisms are able to solubilize the unavailable forms of phosphate through their metabolic activities. The present study concluded that the treatment of physical and chemical mutagenic agents in fungal strains increased the phosphate solubilization efficacy of the fungal strains. This study also strengthened the idea that addition of carbon and nitrogen sources favors the phosphate solubilization to a certain extent.

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