

Isolation and Identification of Phosphate Solubilizing Fungus from Soil

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ABSTRACT

In limiting agricultural production Phosphate (P) plays an important role as one of the major bio-element. Phosphate solubilizing fungi play an interesting role in increasing the bioavailability of soil phosphate for plants. The present study was aimed for isolating and characterizing phosphate solubilizing fungi from rhizosphere using both solid and liquid Pikovskaya (PVK medium). The result showed high solubilising index in agar plate and halo zone was observed around the colony. Phosphate-solubilizing microorganism function in soil phosphorous cycle, increasing the bioavailability of inorganic soil phosphorous for plants. Two fungal colonies were identified as Phosphate Solubilizing Fungus (PSF) and named as AP1 and AP2.

INTRODUCTION

Phosphorus (P) is that the second most vital nutrient for plant growth, accounting for 0.2% (w/w) of plant dry weight. It's second only to nitrogen among mineral nutrients most ordinarily limiting the expansion of crops. Phosphate plays an irreplaceable role within the ecosystem by participating in most aspects of energy metabolism, macromolecule and protein synthesis, and kinase regulation. The typical Phosphorous content in soil is almost 0.05% (w/w) with the most two forms being inorganic Phosphate (Pi) and organic Phosphate (Po). Nevertheless, only 0.1% of P are often utilized by plants, rendering available P a restrictive factor for plant growth. P minerals, like apatites and strengites, have very slow-release rates, other P minerals, complexed with Calcium, aluminium, or iron, have faster dissolution Rates that are hooked in to the pH of the encompassing soil and on the dimensions of the particles. Higher soil pH values (basic) cause aluminium and iron-complexed P to become more soluble, while lower

soil pH values (acidic) promote the solubility of calcium-complexed P. However, the bulk of the applied fertilizer phosphorus isn't available to plants and therefore the addition of inorganic fertilizers in more than the quantity that's commonly employed to beat this effect can cause environmental problems like, groundwater contamination and waterway eutrophication. Consequently, P fertilizers became the most important marketplace for phosphorus worldwide. Thanks to the demand of agriculture on global stocks of Phosphorous, it's estimated that the planet will reach its maximum rate of quality mineral P production by 2040 at which point production will decline while agricultural demand will still rise. Since P supply aren't easily replenished as compared to nitrogen, it's important to raise utilized P reserve within the soil and reclaim chemically-bound P.

Soil microorganisms enhance plant nutrient acquisition. They're involved during a wide selection of biological processes including the transformation of insoluble soil nutrients. Within the natural environment numerous microorganisms within the soil and rhizosphere are effective at releasing phosphorus from total soil phosphorus through solubilization and mineralization. This group of microorganisms are mentioned as Phosphorus Solubilizing Microorganisms (PSM). Many species of soil fungi and bacteria are ready to solubilize phosphorus in vitro and a few of them can mobilize phosphorus in plants. The Phosphate Solubilizing Microorganisms (PSM) play a really important role in phosphorus nutrition by exchanging its availability to plants through release from inorganic and organic soil phosphorus pools by solubilization and mineralization. The main mechanism in the soil for mineral phosphate solubilization is by lowering the soil pH by the microbial production of organic acids by the release of carbon dioxide and mineralization of organic phosphorus by acid phosphates. PSM increases the bioavailability of soil insoluble phosphorus for plant use. They solubilize insoluble inorganic (mineral) phosphorus and mineralize insoluble organic phosphorus. The salt-tolerant or halophilic soil microorganisms that also exhibit the power to solubilize insoluble phosphorus facilitate the event of saline-alkali soil-based agriculture. The beneficial plant-microbe interactions within the rhizosphere are determinants of plant health and soil fertility. Among the rhizosphere microbes, the important genera of P-solubilizing bacteria include *Rhizobium*, *Bacillus*, and *Pseudomonas*. *Penicillium* and *Aspergillus* spp. Are the dominant P-solubilizing filamentous fungi found in rhizosphere. Filamentous fungi are highly important in RP solubilization. They're widely used as producers of organic acid. *Aspergillus niger* and a few *Penicillium* species are tested for solubilization of P and other biotechnological importance like biocontrol, biodegradation, and phosphate mobilization.

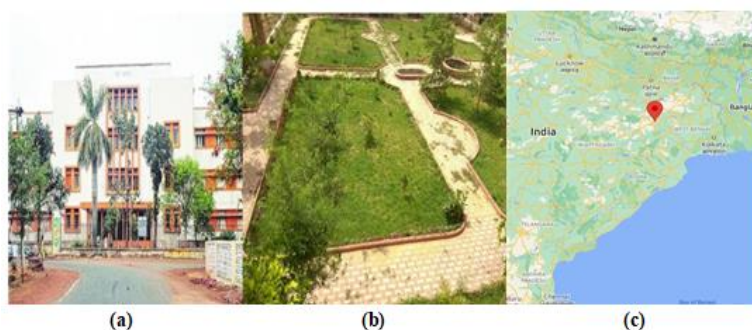
The inoculation of soil or crop with phosphate solubilizing/mineralizing microorganisms is therefore a promising strategy for the development of plant absorption of phosphorus and thereby reducing the utilization of chemical fertilizers that have a negative impact on the environment.

MATERIALS AND METHODS

Collection of soil sample from rhizosphere

Root samples were collected from the college's Botanical Garden of Dr. Shyama Prasad Mukherjee University. It was achieved by digging up soil upto 5-6 cm in depth and the length of the root was approximately 3-4 cm in length. 1 g of soil was taken from the rhizosphere of random plant root. The microbial analysis was conducted in the laboratory of, Department of Microbiology, DSPMU, Morabadi. Geographic coordinate is 23o23'17.8o N in latitude and 85o19'20.6o E.

Figure 1: Sampling site in the DSPMU's Botanical Garden: (a) University campus; (b) Botanical Garden; (c) map showing the location of the study.



Isolation of phosphate solubilizing fungi

To isolate the phosphate solubilizing fungi, Pikovskaya’s media was prepared, sterilized, and dispensed into petri plates. A small amount of soil (1 gm) was taken from rhizosphere of plant sample and serially diluted in the known volume of water. The dilution prepared was upto 10⁻⁵ and each plate was inoculated with 100 µl of sterile soil water suspension of dilution 10⁻² and 10⁻⁴ respectively. Later plates were incubated in for about 5-6 days at 37o C. Composition of the medium was as given in the Table 1 mentioned below. Yeast extract, dextrose, calcium phosphate, ammonium phosphate, potassium chloride, magnesium sulphate, manganese sulphate, ferrous sulphate, agar were some ingredients used in media.

Physical and microscopic identification

Identification of the colony was based on colony characteristics and microscopic features. Observation of surface appearance, texture and colour of the colonies both from upper and lower side were done for physical identification. In addition, conidia, conidiophores, arrangement of spores, vegetative structures were determined with light microscopy using lactophenol cotton blue as staining dye.

Molecular identification

Isolated cultures were sent to Eurofins Genomics India Pvt. Ltd. for DNA extraction and sequencing. The DNA sequencing was done by DNA analyzer.3730xl 96 capillary from applied Biosystems (Life Technologies). Internal transcribes spacer (ITS) 1 (TCC GTA GGT GAA CCT GCG G) and internal transcribed spacer ITS 4 (TCC TCC GCT TAT TGA TAT GC) were used for amplification of internal transcribed region.

Screening of phosphorous solubilizing activity

Both inorganic and organic phosphates exist in soil. Most of the soil fungi and bacteria are phosphate solubilizers and they play a crucial role in maintaining phosphorus balance of crop plants. This fact is exploited in culturing phosphate solubilizers which are able to solubilize bound phosphates. Phosphate as phosphorous is present within the medium and dextrose act as energy source. Different salts and yeast extract support the expansion of organisms. Screening for primary phosphate solubilizing activity of the isolates was carried out by allowing the fungi to grow in selective media that was Pikovskaya’s agar medium and incubated. Later, after incubation period appearance of a transparent halo zone around the fungal colony was observed as positive result.

Determination of Solubilization index on solid medium

Fungal isolates which showed phosphate solubilizing activity as detected on Pikovskaya’s medium by the appearance of halo zones were further subjected for the calculation of solubilizing index. Formula used for determination: ratio of the total diameter (colony +halo zone) and the colony diameter
 Solubilizing index (SI) = colony diameter + clearing zone/colony diameter

Screening of acid production by isolated fungi

Czapek-Dox Agar and Phenol Red Dextrose Agar were prepared and inoculated with fungal isolates. Inoculated plates were incubated at 30°C for 1-2 days. Both the plates were observed after incubation. Talcum powder was used as substitute of calcium carbonate in Czapek Dox agar to observe production of acid.

Table 1. Composition of Czapek Dox agar media in gm/L.

Ingredients	gm/L
Sucrose	30
Sodium nitrate	2
Dipotassium phosphate	1
Magnesium sulphate	0.5
Potassium chloride	0.5

Ferrous sulphate	0.01
Agar	15
Talcum powder	0.5
pH	7.3

Computational analysis of DNA sequences

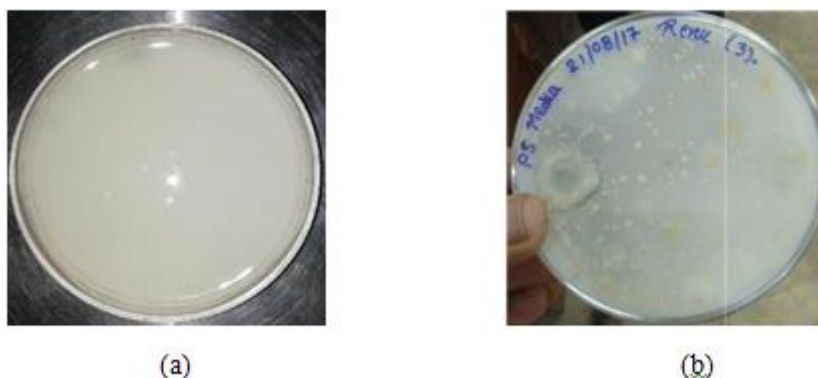
After receiving the sequences in FASTA file from Eurofins, contig assembly was done with the help of DNA dragon a software by Sequentix. Later MEGA X by mega-software was used for the preparation of phylogenetic tree and its analysis.

RESULTS

Isolation of phosphate solubilizing microbes on solid medium

Among mixed cultures 2 fungal isolates were identified that showed phosphate solubilizing activity on Pikovskaya’s medium by the appearance of halo zone around them (Figure 2-4) (Table 2).

Figure 2. (a) Control plate of PVK medium (b) Showing phosphate solubilizing activity around the fungal colony.



Screening and determination of phosphate solubilization by plate assay

$$\begin{aligned} \text{Solubilizing index} &= \text{colony diameter} + \text{clearing zone} / \text{colony diameter} \\ &= 2.65 + 3.15 / 2.65 \\ &= 2.18 \text{ cm} \end{aligned}$$

Solubilizing index of the fungal colony in the given Figure 3 is 2.18 cm.

Figure 3: (a) Control plate of PVK medium (b) Showing phosphate solubilizing activity.



Microscopic identification of fungus isolates

Figure 4. Macroscopic and microscopic characteristics of isolated fungi for phosphate solubilization from DSPMU's Botanical Garden. Isolate AP1: - (a) Colony appearance on PDA; (b) Conidiophore, vesicle, phialides; (c) Septate hyphae; Isolate AP2: - (d) Colony appearance on SDA; (e) Conidiophore, conidiospore; (f) Septate hyphae.

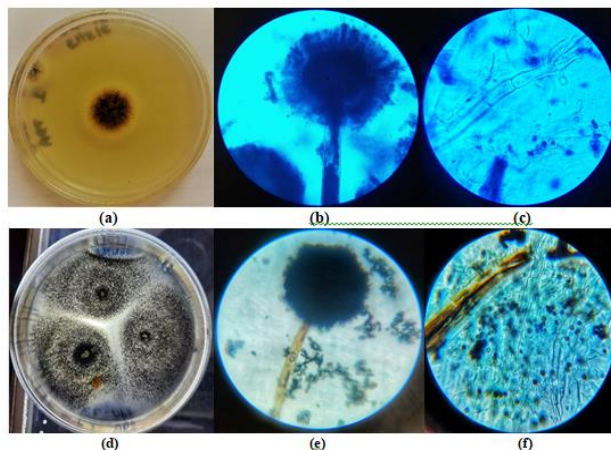


Table 2. Accession number was received by NCBI after submitting sequences of both the isolates.

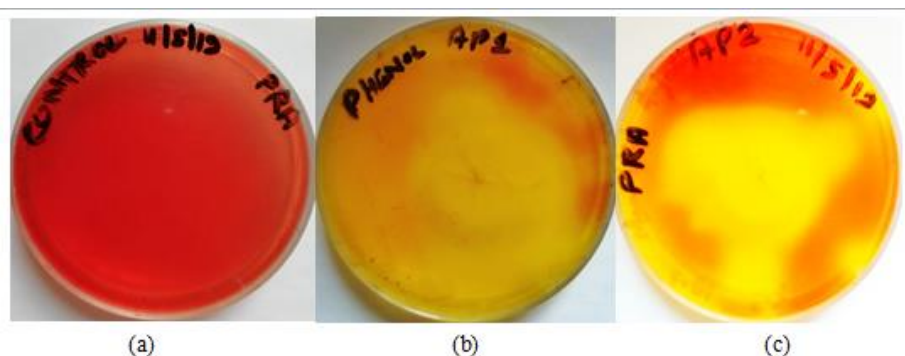
Sr No.	Isolates	Accession Number
1	AP - 1	MK616376
2	AP - 2	MN100313

Molecular identification

Screening of acid production by plate assay on Phenol Red agar medium

Phenol Red Dextrose Agar is suggested for studying the fermentation of dextrose. Proteose peptone which is free from fermentable carbohydrates is added within the agar. With Phenol Red Dextrose Agar, a positive carbohydrate fermentation reaction is indicated by the assembly of a yellow colour the agar, thanks to acid production (Figure 5).

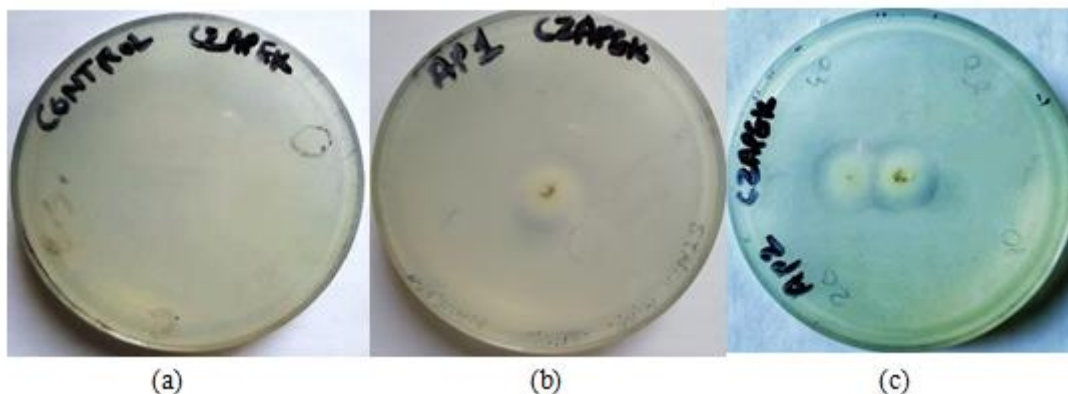
Figure 5: Screening for acid production; (a) Control plate of Phenol Red (b) colour change by isolate AP1 showed acid production; (c) colour change by isolate AP2 showed acid production.



Screening of Citric acid production by plate assay on Czapek-Dox agar medium

Fungi, including yeasts and filamentous species or moulds are ubiquitously distributed in nature. Czapek Dox Agar is a semisynthetic medium used for the cultivation of fungi, containing sodium nitrate as the sole source of nitrogen and a small amount of calcium carbonate was used for the detection of acid production. Acid production show halo zone around the colonies which indicates the production of acid by degrading calcium carbonate present in talcum powder. Talcum powder used in place of calcium carbonate didn't consist of antimicrobial properties.

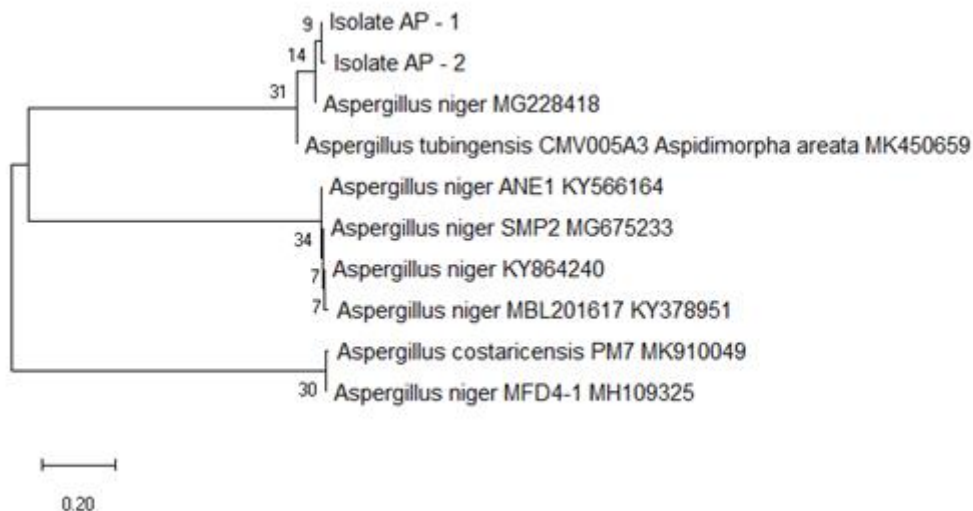
Figure 5: Screening for acid production; (a) Control plate of Czapek-Dox Agar (b) halo zone formed by isolate AP1 showed acid production (c) halo zone formed by isolate AP2 showed acid production



Computational analysis of DNA sequences

Subsequently the sequences result of ITS genes of our study are aligned with the corresponding sequences of recorded fungal organisms, and the resulting phylogenetic tree revealed that these isolates were clustered into *Aspergillus spp.* Similarity percentage was very closely related over 99%. FASTA sequences for 2 samples obtained from DNA sequencing were used to identify the species through BLAST. All sequences retrieved were having Expected (E) value 0.0, which means the matches were significant. The trees scale bar represents a 0.20% difference of fungal isolates AP1 and AP2 in nucleotide sequences and it's obviously from the tree that the isolates clustered with their specific matches from the database (Figure 6).

Figure 6: A phylogenetic tree for fungal isolate AP1 and AP2 created by MEGA X.



DISCUSSIONS

Phosphate Solubilizing Fungi (PSF) are beneficial fungi capable of solubilizing inorganic phosphorus from insoluble compounds. Phosphorus (P) is one of the major essential macronutrients for plants and is applied to soil in the form of phosphate fertilizers. However, a large portion of soluble inorganic phosphate which is applied to the soil as chemical fertilizer is immobilized rapidly and becomes unavailable to plants. We carried out phosphate solubilization screening of two fungal strains in solid PVK media in vitro. This study indicates that the AP1 and AP2 were efficient in solubilizing phosphate. Isolate AP1 and AP2 also showed the greatest drop in pH in the PVK broth containing Tricalcium Phosphate (TCP), suggesting that it might produce organic acids. Our results are similar to the study of which concluded that *Aspergillus spp.* Show a much higher drop in pH in the PVK broth containing TCP and high P solubilization when compared to *Penicillium spp.* Phosphate-solubilizing microorganisms convert insoluble phosphates into a soluble form through the processes of acidification, chelation, exchange reaction, and production of organic acid.

Bacteria, fungi, actinomycetes and even algae play an important role in P solubilization. The other mechanism is that the production of H₂S, which react with ferric phosphate to yield ferrous sulphate with concomitant release of phosphate. The mechanism of P solubilization that's employed mostly by soil microorganisms includes: (1) release of complex compounds e.g., organic acid anions, protons, hydroxyl ions, CO₂, (2) liberation of extracellular enzymes or it also referred as biochemical P mineralization and the release of P during the degradation of substrate. Thus, microorganisms have key role within the soil P cycle i.e., precipitation and mineralization. PSMs apply various approaches to form phosphorus accessible for plants to soak up. These include lowering soil PH, chelation, and mineralization.

The present study revealed that the soil supports group of P-solubilizing microbes. The most dominant genera belong to *Aspergillus spp.* Similarly, also isolated P-solubilizing fungi such as *Aspergillus niger* and *Penicillium spp.* from various rhizospheric soil samples. The effectiveness of P-solubilizing fungi in the present study could be probably because of sufficient root exudates since phosphate solubilizing microorganisms are mainly dependent on carbon rich sources from plant root for active production of organic acids that are utilized for solubilizing soil-bound phosphate. In the present study, *Aspergillus spp.* (55.69%) were the most frequently occurring P-solubilizing fungi of the three identified genera. This may be due to the efficiency of *Aspergillus sp.* in root colonization. Acidification by organic acid has been reported to be the main solubilization mechanism of inorganic P by microorganisms so, for confirmatory test we used Phenol red and Czapek-Dox agar as medium to observe acid production. Observation showed positive result for acid production on both the medium.

There was a time when chemical fertilizers were used rigorously, though production was high but it decreased quality of the soil. Due to the adverse effect of chemical fertilizers agricultural sector started switching over to biofertilizers. In the series of developing biofertilizers microorganisms played an important role through various aspects. As phosphorous is second most dominant element for plant growth so scientists started working in regard to solubilizes and immobilize phosphorous for plant growth. Later these phosphate solubilizing microbes became a sustainable approach for managing phosphorus deficiency in agricultural soils. Today also studies are going on how these microbial inoculants can be used for solubilizing and immobilizing phosphorous that will help in growth of the plants.

Applications of beneficial microbes, especially phosphate-solubilizing fungi, are useful toward plant growth-promoting strategies. The isolate AP1 and AP2 were highly efficient strain in solubilizing phosphate; accordingly, we are optimistic that it can play potential roles in supporting plant growth in practical applications. This will have critical implications for the development of agriculture, industry, restoration ecology and forestry going forward.

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