Optimizing Phosphorus Solubilization Through Rhizosphere-Isolated Fungi

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Abstract: Phosphate Solubilizing Microorganisms (PSMs) are capable of solubilizing insoluble forms of soil phosphate to liberate soluble P and make it available to plants. The growth and productivity of many different crops can be enhanced through these PSMs. Thus, introducing PSMs to seeds, crops, and soil is promising for promoting sustainable agriculture without endangering the environment. Hence this study was conducted to isolate and optimize the culture media for effective P solubilizing fungus. All experiments were conducted in laboratory conditions with a Complete Randomized Design (CRD) with three replicates. All the data were statistically analyzed using analysis of variance (ANOVA), and means were separated using Tukey's HSD test. Four fungal strains were isolated from different plant rhizospheres of rice (1), maize (1), and chilli (2) plants as potential P solubilizers which produced a clear halo appearance. These fungal strains were tested for their capability on P solubilization by using phosphorus solubilizing index (PSI) in plates and the amount of solubilized P in broth cultures to identify the most effective P solubilizer. The selected fungus was further tested for media optimization. Different nitrogen (N), phosphorus (P), carbon (C) sources, and pH levels were optimized under in vitro conditions. One of the fungi isolated from chilli rhizosphere (Fd) was identified as the most effective P solubilizer with an established significantly highest solubilized P (2.54 ppm ± 0.13) and the average highest PSI (1.68). The media optimization study results showed that potassium di-hydrogen phosphate as P source, fructose as the C source, ammonium sulphate as the N source, and neutral pH (pH 7) could maximize the solubilization of P with Fd. Further improvements would be essential to introduce Fd as a P-solubilizing fungal inoculum to the soil.

Keywords: *Media optimization, Phosphorous solubilizing microbes, Phosphorous solubilization, media optimization, Rhizosphere*

1. INTRODUCTION

Most tropical soils are rich in various insoluble forms of Phosphorous (P) but less in available forms which plants can readily absorb (Gyaneshwar *et al*., 2002). For over one hundred years, scientists have recognized the ability of soil microorganisms to solubilize insoluble forms of organic and mineral phosphates into available forms (Whitelaw, 1999). These microorganisms inhabit the rhizosphere of plants probably as symbiotic associations (Abeysingha and Weerarathne, 2010) and have higher metabolic activity than other microorganisms (Vazquez *et al*., 1998).

Microorganisms are an essential component of soil, and their helpful or harmful actions directly or indirectly influence soil health (Abeysingha and Weerarathne, 2010). Rhizospheric microorganisms have a role in soil activities like decomposition and nitrogen cycling, solubilization and mineralization, nutrient storage and release and water, as well as nitrogen fixation and denitrification. Furthermore, organisms with the ability to dissolve P can also turn insoluble phosphatic chemicals into soluble ones, which develops in soils allowing them to be used by the crops. Rhizospheric function microorganisms were involved in mineral phosphate solubilization early as 1903 (Khan *et al*., 2010).

The metabolic activities of microbial populations are important. Plant growth can be

aided by rhizosphere microbes such as fungi via various processes. One of these is the breakdown of insoluble P, whose soil availability is regulated by pH values, allowing P to be absorbed by plants (Khan *et al*., 2010). Phosphate solubilizing microorganisms (PSM) are soil microbes that may dissolve insoluble forms of phosphates into plant-available forms as part of the P cycle. Fungi, bacteria, and actinomycetes are among the microorganisms that are known to be efficiently fixed P solubilizers (Sundara et al., 2002). The population of phosphate solubilizing microorganisms is more in the rhizosphere (20-40 % of the total population) as compared to the non-rhizospheric (10-15% of the total population) region (Swaby and Sperber, 1958).

P-solubilizing fungi (PSF) have been reported to possess a more remarkable ability to solubilize insoluble phosphate than bacteria (Whitelaw, 1999). Among PSMs, fungi perform better in acidic soil conditions. Among the rhizosphere microbes, the important genera of bacteria including Pseudomonas, Bacillus, and endosymbiotic rhizobia have been described as effective phosphate solubilizers (Seema *et al*., 1997). Alternaria, Arthrobotrys, Aspergillus, Fusarium, Glomus, Micromonospora, Penicillium, and Saccharomyces have been identified as effective genera of fungal P solubilizers (Srinivasan *et al*., 2012). The primary objective of this study is to isolate potent phosphate-solubilizing fungi from rhizospheric soils and develop an optimized media formulation for efficient phosphate solubilization, focusing on potential industrial applications.

2. METHODOLOGY

2.2.1 Soil Sample Collection

Three soil samples were collected from rice, maize, and chilli rhizospheres which belongs to the great group Reddish Brown Earth (RBE) (Panabokke, 1996) soils, Anuradhapura, Sri Lanka.

2.2.2 Isolation, Screening of P Solubilizing Fungus/ Fungi in PVK Agar

Phosphorus-solubilizing fungi were isolated from each soil sample using ten-fold serial dilutions and spread plates. Five grams (5g) of each soil sample were dispersed in 10 ml of autoclaved distilled water and thoroughly shaken. From this solution, 1 ml was transferred to 9 ml of sterile distilled water to create a 10^{-1} dilution. Similarly, 10^{-2} and 10^{-3} serial dilutions were prepared for each rhizosphere soil.

Next, 100 microliters of 10^{-3} dilution were spread on Potato Dextrose Agar (PDA) media separately and three replicates were maintained for each sample. The plates were then incubated at 28 ± 1°C for 3-7 days. After incubation, four fungal colonies were successfully isolated as: Rice (Fig. 1a), Maize (Fig. 1b), and Chilli (Fig. 1c and d). To obtain pure cultures, the isolates were further purified by sub-culturing multiple times on the same medium.

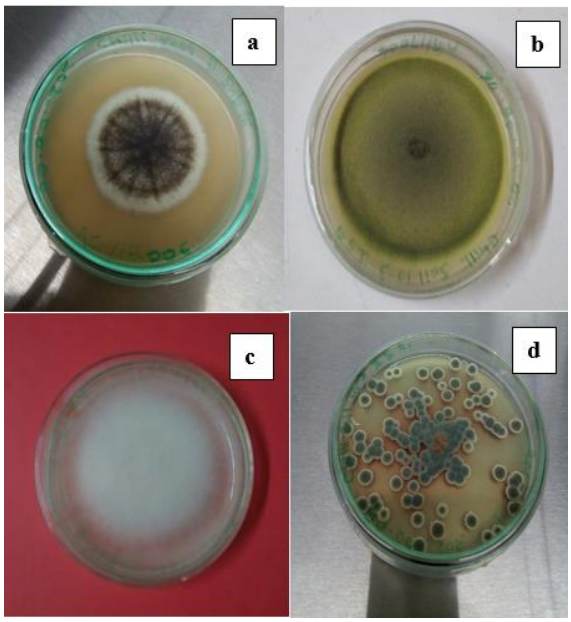

Figure 1: Initial colony morphologies of fungi isolated from different rhizospheres; (a) Rice, (b) Maize, and (c)-(d) Chilli

All isolated fungi were grown rapidly at 25-30°C and covered the surface of the PDA. Figure 2 shows the colonies of fungi "c". Initial stage colony appearances were whitish in color, and older colonies appeared grey to brown in color. This particular colony displayed notably rapid

growth compared to the other species. Based on our observation of its morphology, it exhibits typical characteristics consistent with the genus *Mucor* within the family *Mucoraceae.* Some of the key morphological features observed mainly include broad, dense, cotton like mass with a white to greyish color mycelium. The colony morphology of fungi "b" appeared as powdery masses of yellowish-green spores on the upper surface and reddish-gold on the lower surface, which closely resembled the typical morphology of *Aspergillus spp*. within the family *Trichocomaceae*.

The colony morphology of fungus "a" also exhibited similar features to the genus *Aspergillus*. Initially, the colonies appeared as white in color and gradually turned black over time. During the initial stage, the colonies displayed an upper dark greenish color, which changed to yellowish-brown as they matured, resembling the characteristics of fungus "d" as *Penicillium*. (Figure 2).

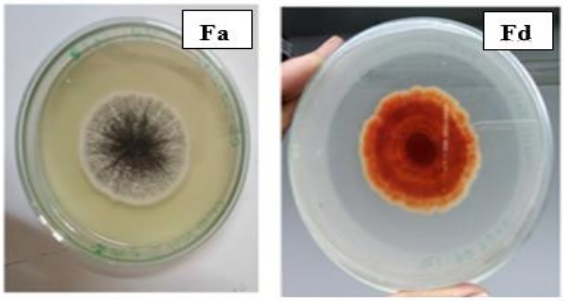

Figure 2: Older colony morphologies of fungus "a" and "d"

2.2.3 Determination of P solubilizing index (PSI) on Pikovskaya (PVK) agar medium

Fungi produced a clear halo appearance in PVK agar solid media, which were incubated at 28 ± 1^0C for 3-7 days, and the highest PSI were selected as effective P solubilizer. The diameter of clear zones around the colony of each isolate was measured after the 7^{th} days of incubation. PSI was calculated (Premono *et al.*, 1996).

2.2.4 Evaluation of the Efficiency of Phosphate Solubilization in PVK broth

Sporulated pure fungal cultures prepared on PVK solid medium were selected to prepare spore uspensions from each fungal isolate. A total volume of 20 ml of sterile water was spread in aliquots on a culture plate, and the fungal colony surface was lightly scraped using a sterile spreader. Subsequently, 50 ml of PVK liquid media was inoculated with spores taken from a particular fungal strain, while a control was maintained without spores. Three replicates were maintained for each test fungus. All the samples were kept in a shaker for 24 hours at 100 rpm at room temperature. After incubation, media was filtered through Whatman No. 42 filter paper and centrifuged at 7200 rpm for 20 minutes to remove suspended solids and mycelial fragments (Kumari *et al.*, 2010). Supernatants were collected to estimate the amount of solubilized phosphorus concentration using the Murphy and Riley method (2002), and the remaining suspension was used to measure the pH values.

2.2.5 Identification of effective P solubilizer

The highest PSI and solubilized P resulted in the most effective P solubilizer among four isolated fungal strains.

2.2.6 Optimization of media for phosphorus solubilization

Different sources of N, P, C, and three different pH levels were tested to optimize the media for higher solubilization of P with selected P solubilizer.

P sources; potassium dihydrogen phosphate [KH_2PO_4], sodium phosphate [Na_2PO_4], rock phosphate, tri-calcium phosphate [$Ca_3\ (PO_4)_2$], N sources; ammonium sulphate [$(NH_4)_2SO_4$], urea, and sodium nitrate [$NaNO_3$], C sources; glucose, fructose, and sucrose and pH 5,6,7, variations were studied in PVK Broth.

The effective isolate was checked for solubilization activity in PVK broth, amended with different nutrient sources, and maintained

pH levels. Three replicates were carried out for each phosphate source. Flasks were incubated at 37^0C for four days (Fasim *et al.*, 2002). After incubation, media was filtered through Whatman No. 42 filter paper and centrifuged at 7200 rpm for 20 minutes to remove suspended solids and mycelial fragments. Supernatants were collected, and 0.5 ml of each culture was then taken out to estimate the amount of solubilized phosphorus concentration using Murphy and Riley method (2002), and the remaining suspension was used to measure the pH values.

2.2.7 Statistical analysis

The experiment data were subjected to ANOVA tests, and Tukey's HSD test was used to separate the means using SAS software.

3. RESULTS AND DISCUSSION

3.1 Efficiency of Phosphorus Solubilization

Four fungal strains were evaluated for their phosphate solubilizing efficiency using PSI (Table 1).

Table 1: PSI for isolated fungal strains

Fungi	Isolated rhizosphere	Average PSI ± SD
a	Rice	1.36±0.06
b	Maize	1.03±0.01
c	Chilli	1.11± 0.04
d	Chilli	1.68± 0.08

Fungus "d" exhibited the highest average PSI (1.68± 0.08), indicating superior P solubilization compared to other fungi. Fungus "d" belongs to one of the largest and most fascinating groups of fungi, namely *Penicillium spp.*, which are well-known for their presence in diverse environments ranging from saline soils to arctic regions. *Penicillium* species have been reported in numerous terrestrial environments.

3.2 Efficiency of phosphorus solubilization in PVK liquid medium

The excretion of organic acids has often been associated with microbial solubilization of soil P. The role of organic acids produced by PSM in solubilizing insoluble phosphate may be attributed to several factors, including the lowering of pH, chelation of cations, and competition with phosphate for adsorption sites in the soil (Nahas, 1996). Previous evidence suggests that different organic acids are produced during the solubilization process. However, it is important to note that acidification does not appear to be the sole mechanism of solubilization. In some cases, the reduction in pH did not correlate with the ability to solubilize mineral phosphates (Subba Rao, 1982).

In this study, the reduction of pH in the culture media was synchronized with the process of P solubilization, as evidenced by the lowest pH value (4.15 ± 0.22) recorded in the medium containing the fungus "d", which also exhibited the numerically highest solubilized P. However, this difference did not show any statistical significance compared to the results obtained with fungus "a" (Figure 3).

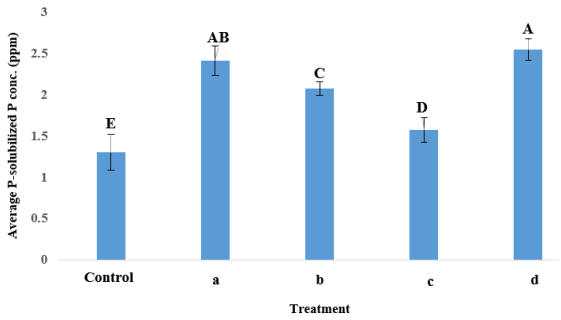

Figure 3: Solubilized P in PVK liquid medium. Different letters indicate significant differences at a 5% probability level based on Tukey's mean comparison test.

3.3 Effective P solubilizer

The fungus isolated from the Chilli rhizosphere, designated as "d", was identified as the most effective phosphorus solubilizer. This determination was based on its highest PSI value

and its ability to solubilize the most phosphorus in the PVK medium.

3.4 Media Optimization for Solubilization of P by isolation of "d"

Different sources of N, P, C, and three different pH levels were tested to optimize the PVK media for higher solubilization of P with fungus "d".

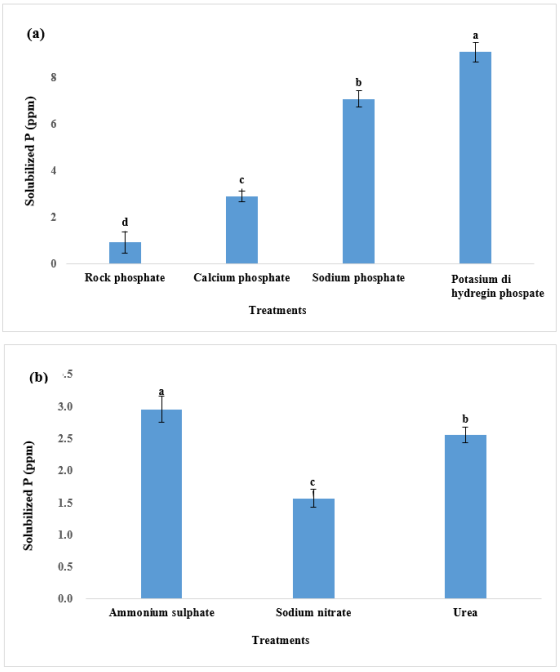

Figure 4: Effect of various P sources (a) and N sources (b) on the efficiency of P solubilization. Statistically significant differences at a 5% probability level are indicated by different letters, according to the Tukey's mean comparison test.

The effect of various P sources, such as Rock phosphate (RP), Tri calcium phosphate [$Ca_3(PO_4)_2$], Sodium phosphate [Na_3PO_4], and Potassium dihydrogen phosphate [KH_2PO_4], showed that KH_2PO_4 was the best phosphate source, with a solubilization rate of 9.081 ppm. Next in line was Na_3PO_4, which exhibited moderate P solubilization, whereas $Ca_3(PO_4)_2$ and RP demonstrated minimal P solubilization. While investigating the impact of different N sources on P solubilization, it was discovered that ammonium sulphate $(NH_4)_2SO_4$ exhibited the highest P solubilization, followed by urea. This finding aligns with previous reports indicating that many fungi and bacteria can solubilize phosphate effectively only in the presence of ammonium as the nitrogen source (Illmer et al., 1995).

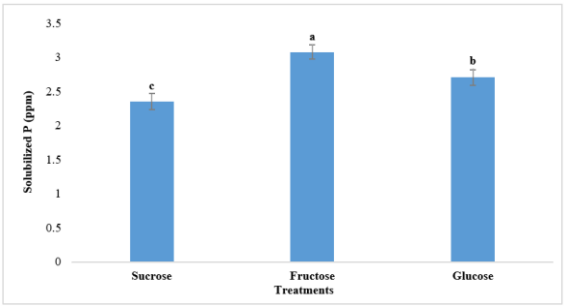

Figure 5: Effect of various C sources on the efficiency of P solubilization. Different letters indicate statistically significant differences at a 5% probability level according to the Tukey's mean comparison test.

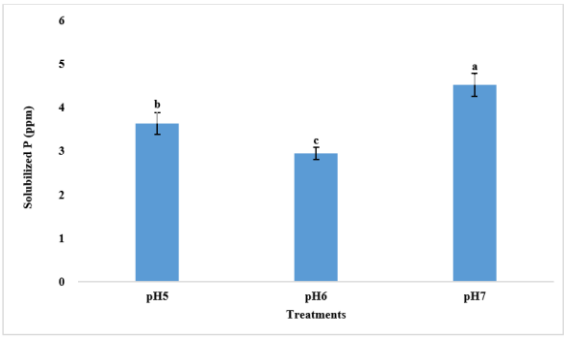

Figure 6: Effect of different pH levels on the efficiency of P solubilization. Different letters indicate statistically significant differences at a 5% probability level according to the Tukey's mean comparison test.

When various carbon sources were used to study the P solubilization, it was found that PVK with fructose gave maximum P solubilization, followed by glucose and sucrose. Microorganisms' solubilization ability is related to the nature of the acid produced (Vassileva *et al*., 2001). Some bacterial isolates have solubilized P only in the presence of fructose and co-solubilization in glucose and sucrose (Fasim *et al*., 2002) (Figure 5).

The pH is the vital factor in solubilization; in most cases, P solublization is the result of organic acid production. The results showed that the maximum P solubilization was monitored at pH 7 (Figure 6).

4. CONCLUSION

Out of the four isolates tested, the fungus isolated from Chilli rhizosphere was identified as an effective P solubilizer based on PSI in PVK solid media and its efficacy on PVK liquid media. The results of media optimization tests revealed that using potassium dihydrogen phosphate as the P source, fructose as the C source, ammonium sulphate as the N source, and maintaining a neutral pH (pH 7) resulted in the highest solubilization of P when using modified PVK media with different nutrient sources. Moreover, this effective solubilizer demonstrated great potential for developing an inoculum for soil when using its optimized media. However, further improvements would be essential before introducing Fd as a P-solubilizing fungal inoculum.

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