Isolation and Characterization of Phosphate Solubilizing Microbes from Rock Phosphate Mines and Their Potential Effect for Sustainable Agriculture

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Abstract: Continuous application of phosphate (P) mineral to soil renders apatite addition during each crop growing season which is of great concern from a sustainable agriculture viewpoint. Use of efficient phosphate solubilizing microbes (PSB) is one of the most effective ways to solubilize this apatite mineral in the soil. The current study targeted hydroxyapatite mines to explore, isolate, and characterize efficient P solubilizers to solubilize apatite in the soil. Efficiency of isolated microbes to solubilize rock phosphate (hydroxyapatite) and tri-calcium phosphate (TCP) as well as indole-3-acetic acid (IAA) and 1-aminoacyclopropene-1-carboxylate deaminase (ACC) activity were tested. Identification and phylogenetic analysis of bacterial and fungal isolates were carried out by 16s rRNA and internal transcribed spacer (ITS) rDNA sequence analyses, respectively. The isolated bacterial strains were identified as Staphylococcus sp., Bacillus firmus, Bacillus safensis, and Bacillus licheniformis whereas fungal isolates were identified as Penicillium sp. and Penicillium oxalicum. Results showed that the impact of identified strains in combination with three phosphate fertilizers sources (compost, rock phosphate and diammonium phosphate (DAP)) was conspicuous on maize crop grown in pot. Both bacterial and fungal strains increased the P uptake by plants as well as recorded with higher available P in post-harvested soil. Penicillium sp. in combination with compost resulted in maximum P-uptake by plants and post-harvest soil P contents, compared to other combinations of P sources and bio-inoculants. Screening and application of efficient P solubilizers can be a better option to utilize the indigenous phosphate reserves of soil as well as organic amendments for sustainable agriculture.

Keywords: rock phosphate; solubilization; isolation; characterization; maize; compost; DAP

1. Introduction

Today the major source of phosphorus (P) fertilizer comes from phosphate rock (PR) which is a finite resource like oil and gas reserves. Global potential P reserves were...
estimated to be around 11–22 billion tons. According to the United State Geological Survey, global PR reserves are more than 300 billion tons. It has been predicted that 70% of current global reserves will be depleted within 100 years and, considering the increase in global demand, a significant global production deficit is predicted by 2070. Phosphorus plays a significant role in photosynthesis, respiration, formation of cell membranes, glycolysis and enzymes activities of plants [1]. In plants, it increases root development, empowers stem, improves seed formation, increases crop maturity and nitrogen fixation [2]. About 90% of the phosphate demand is used for food purposes. Phosphate fertilizer applied to the soil is taken away with harvest crops and its consistent supply is crucial to sustain the world food supply. Unlike nitrogen (N), when applied to the soils P is immediately fixed in soil and its availability to plants reduces even after hours of application. Based on soil type, applied P interacts with iron (Fe), Aluminum (Al), calcium (Ca) and other soil organic compounds which render its availability to plants. Its re-dissolution in soil and bioavailability largely depends on the soil type, cultural practices and soil microorganisms. Phosphorus fixed with calcium or aluminum oxides is sensitive to pH changes, while Fe-P is also sensitive to reducing conditions. Up to 80% of the applied P is fixed in soil and release of this huge amount of fixed P pool is of great concern from a viewpoint concerned with efficient use of this finite resource. Furthermore, production of chemical P fertilizer from rock phosphate is also a highly energy intensive process, consuming 17,500 kJ/kg energy costing over four billion USD each year to meet the global demand. Therefore, it is important to devise such strategies which can not only help to transform unavailable soil P pools to labile form but also help in solubilizing P from rock phosphate when applied to the soil.

Use of phosphate solubilizing microorganisms is a potential area as these microorganisms contribute in soil P cycle [3]. Microorganisms can enhance soil fertility through decomposition, mineralization, microbial storage and release of nutrients in soil. They may be considered inexpensive options for increasing P availability to plants [4]. Naturally occurring phosphate solubilizing microbes have been reported since 1903. Phosphate solubilizing microbes (PSMs) have been reported to have a direct relation with plant growth which provides a reason to select the most promising microbes for better crop growth and development [5]. Soil microorganisms release low molecular weight organic acids that weather insoluble rock phosphate and tricalcium phosphate, and increase P availability for plant uptake [6,7].

Similarly, many fungal strains have also been reported as phosphate solubilizers but the most dominant phosphate solubilizing fungi include *Aspergillus niger* and *Penicillium* sp. [8]. Inoculation with mycorrhizal fungi can reduce the use of phosphate fertilizers up to 80%, producing optimum growth and yield [9]. Phosphate solubilizing microbes are involved in key processes such as soil structure formation, decomposition of organic matter, toxin removal, and the cycling of elements like carbon, nitrogen, P, potassium and sulfur [10]. In recent years, phosphate solubilizing fungi have gained much attention because of their high solubility with the phenomenon of releasing organic acids even in extreme weather conditions. In addition to phosphate solubilization, microbes are capable of producing phytohormones that can promote plant growth [11].

The identification of isolated phosphate solubilizing fungal and bacterial strains at a molecular level is helpful to open up new avenues of research and development. For eukaryotic organisms, 18S rRNA serves as a conserved region that may be amplified through polymerase chain reaction [12]. Many attempts have been made to isolate and identify endophytic and rhizospheric microorganisms and their potential to solubilize insoluble soil phosphate. However, exploring most efficient phosphate solubilizers from the soil layer present in apatite reserves of phosphate mines is a relatively new area of research. The current study is therefore focused on the isolation of phosphate solubilizing microbes from hydroxyapatite reserves of Hazara mines, Pakistan. The isolates showing high P solubilizing abilities were identified through molecular characterization and were used as bio-inoculants in combination with three phosphate fertilizer sources to determine their effect on maize growth.
2. Materials and Methods

2.1. Sample Collection and Microbial Isolation

Soil samples were collected from soil present in Tarnawai and Kakul mines of RP reserves. Samples were carefully collected in zip lock plastic bags and stored at 4 °C.

Isolation of phosphate solubilizing microbes from soil collected rock phosphate mines was done with Pikovskaya’s medium [13]. Colonies displaying clear halo zones were selected and re-streaked until pure cultures were obtained. The purified colonies were maintained in glycerol stocks while fungus strains were maintained on potato dextrose agar (PDA) slants for further studies. Spot inoculation of purified colonies was performed on Pikovskaya’s agar plates containing tricalcium phosphate (3 g L⁻¹) and incubated at 28 °C (±2) for five days. The solubilization index (SI) of each isolate was recorded after seven days using the following equation:

Available phosphorus was determined by phospho-molybdate blue color method [14]. For estimates of indole-3-acetic acid (IAA) production in bacterial and fungal cultures, HPLC method was adopted as proposed by Priyadharsini and Muthukumar [15]. Bacterial and fungal cultures were grown in LB and CZ broth, respectively with or without tryptophan (0.1 g L⁻¹) and incubated at 30 °C for 7 days. IAA was quantified with high performance liquid chromatography (HPLC; Agilent 1100, Waldbronn, Germany) equipped with a UV detector with absorbance at 280 nm and C-18 column (39 × 300 mm). Mobile phase; Methanol: water (80:20; v/v) was used at a flow rate of 1.5 mL min⁻¹. Sample volume (10 µL) was injected using a sample injector. Analyte peaks were compared with internal standards being added to the medium. Dworkin and Foster (DF) salt minimal media (5 mL) was used for determination of 1-aminocyclopropane-1-carboxylate deaminase (ACC) deaminase production of isolated strains. The treatments were: (1) no ACC and no inoculant (control), (2) ACC and no inoculant, (3) no ACC + inoculant and (4) ACC + inoculant. Tubes were shaken for 24 h under 37 °C and the turbidity in the media was observed. Turbid media in ACC treatments suggested microorganisms were utilizing nitrogen as a source.

2.2. Characterization of Isolated Microbial Strains

Complete bacterial genomic DNA was extracted by the CTAB method of genomic DNA extraction. While fungal DNA was extracted according to the method proposed by Cenis (1992). Bacterial 16S rRNA gene and fungal ITS (internal transcribed spacer) rDNA regions amplification was done through PCR, and sequence analysis was done. Extracted bacterial 16S rRNA genes were amplified through 16S primers fD1 (5′-AGAGTTTGATCC TGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′). ITS rDNA regions of fungal isolates were amplified using universal primers of ITS1 (5′-TCCGTAATGACGCTCAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR amplification was carried out in 0.2 mL PCR tubes. Taq polymerase (Thermo Fisher Scientific) at 5 U/µL was used. A 96-well thermal cycler (MultiGene™, Labnet International Inc. North America) was used in PCR. Reaction volume was 25 µL and contained 10x buffer (2.5 µL), 25 mM MgCl₂ (2 µL), 2 mM dNTP (2.5 µL), 10 µM primers (1 µL each), Taq polymerase DNA (0.375 µL) and Template DNA (2.5 µL). The PCR reaction was carried out with 35 cycles at initial denaturation temperature of 95 °C for 5 min, denaturation 95 °C for 1 min, annealing 50 °C for 1 min, extension 72 °C for 1.5 min, and final extension at 72 °C for 5 min. PCR amplified products were checked by electrophoresis on 1% agarose gel. A 5 µL of the PCR amplified sample was loaded on gel by diluting it in 1 µL 6X DNA loading dye. A 1 kb ladder (Thermo Fisher Scientific, Carlsbad, CA, USA) was also loaded on gel with samples to estimate the size of PCR amplified product. Electrophoresis of gel was performed for 45 min at 120 volts and ethidium bromide-stained gel was viewed on a UV illuminator using the gel documentation system In Genius3 (Syngene, USA) operated by GeneSys software, image captured and saved.

Sequences of bacterial and fungal isolates were analyzed by BLAST (Basic Local Alignment Search Tool) and compared with similar sequences available in nucleotide databases.
Molecular evolutionary genetics analysis MEGA 7 software was used for phylogenetic analysis [16]. Multiple nucleotide sequences were aligned by ClustalW and phylogenetic trees were constructed by using a neighbor-joining algorithm [17]. Bootstrapping technique was used for confidence limits with 1000 repetitions. Pairwise percent similarities between all bacterial and fungal isolates were determined using MegAling application of DNA Star (Lasergene software package).

2.3. Effect of Phosphate Solubilizing Microbes on Maize Growth (Pot Experiment)

A pot experiment was conducted at Agricultural Research Farm, The University of Haripur, Pakistan under greenhouse conditions to evaluate the effect of PSMs on phosphorus phytoavailability and its impact on growth and development of maize crop. Soil used in this study was collected from the agricultural research farm of the university from 0–15 cm depth. Soil was air dried, sieved (2 mm) and a composite sample was taken from the soil heap for physico-chemical analysis (Table 1). Soil was silty clay loam (sand 20%; silt 30; clay 50); 4 kg soil was mixed with respective inoculum and added in plastic pots (18 × 23 cm²). On the basis of phosphate solubilizing ability, two fungal and two bacterial isolates were selected for the purpose. Seeds of maize variety ‘Sarhad’ were surface sterilized by washing with 2% sodium hypochlorite and 70% ethanol for 2 min. Then seven seeds were sown in each pot which were thinned to four seeds after germination. Experiment contained 15 treatments having four PSMs; PSM₁ = S₅F₁ (Penicillium spp.), PSM₂ = S₃F₁ (Penicillium oxalicum), PSM₃ = S₁₋₂ (Staphylococcus sp.), PSM₄ = S₅-imp (Bacillus licheniformis) and three P sources; vermizote compost (1.14.5% P), rock phosphate (28% P₂O₅) and DAP (46% P₂O₅) each at the rate of 80 kg ha⁻¹. Crop was harvested 5 weeks after sowing. Root fresh mass (g), root dry mass (g), maximum root length (cm), shoot dry biomass (g plant⁻¹), P uptake (mg plant⁻¹) and total P (mg g⁻¹) in post-harvest soil were determined using standard methods.

Table 1. Pre-sowing analysis of soil used in pot experiment.

| Soil Characteristic | Unit | Value |
|---------------------|------|-------|
| Soil Textural Class | Silty clay loam |
| pH                  |      | 7.81  |
| EC                  | (dSm⁻¹) | 1.21 |
| Organic matter      | (%)  | 0.56  |
| HCO₃                | (%)  | 5.79  |
| P(Extractable)      | (ppm) | 5.7   |
| K(extractable)      | (ppm) | 77    |

EC-Electrical conductivity, HCO₃-Bicarbonate, P-Phosphorus, K-Potassium.

2.4. Statistical Analysis

Pot experiment was laid down in completely randomized design (CRD) two factorial design. The obtained data were statistically analyzed by Statistics 8.1 statistical software, analysis of variance techniques and treatment means were compared using the Tukey’s honestly significant difference (HSD) Test at 0.5% (p ≤ 0.5) probability level [18].

3. Results

3.1. Qualitative Measurement of Phosphate Solubilization

Different bacterial and fungal isolates were tested for phosphate solubilization. Among the bacteria, only three isolates showed phosphate solubilization index (PSI) > 1.5. Isolate S₅-imp showed the highest PSI value (1.94) followed by S₁₋₂ (1.86) and S₅₋₃ (1.66) while the remaining four isolates, including S₅₋₄, S₇₋₁ and S₂₋₂, showed PSI < 1.0 (Table 2). Among the fungal isolates, S₅₋₁ showed the highest PSI value of 4.33 followed by S₅₋₂ (4.0) and S₄₋₁ (3.5), while S₃₋₁ showed a PSI value of 3.0 (Table 2).
Table 2. Biochemical characterization of isolated bacterial and fungal strains.

| Isolate | Category | Zone Formation | Solubilization Index | IAA Production | ACC Activity | Phosphorus from TCP (ppm) | Phosphorus from Rock Phosphate (ppm) | TCP Media pH 7.0 |
|---------|----------|----------------|----------------------|---------------|--------------|--------------------------|-------------------------------------|-----------------|
| S2–1    | Bacteria | ++             | 1.57                 | ++            | ++           | 176.09                   | 88.67                               | 5.25            |
| S5–4    | Bacteria | ++             | 0.36                 | ++            | ++           | 33.67                    | -                                    | 3.44            |
| S7–1    | Bacteria | ++             | 0.61                 | ++            | ++           | 60.82                    | -                                    | 4.4             |
| S1–2    | Bacteria | ++             | 1.86                 | ++            | ++           | 273.74                   | 156.52                              | 4.44            |
| S5–3    | Bacteria | ++             | 1.66                 | ++            | ++           | 193.45                   | 67.91                               | 5.57            |
| S2–2    | Bacteria | ++             | 0.71                 | ++            | ++           | 77.41                    | -                                    | 5.29            |
| S5–imp  | Bacteria | ++             | 1.94                 | ++            | ++           | 247.70                   | 111.45                              | 5.07            |
| S6–F1   | Fungi    | ++             | 3                    | ++            | -            | 387.52                   | 693.16                              | 2.8             |
| S6–F2   | Fungi    | ++             | 4.33                 | ++            | -            | 394.76                   | 542.70                              | 2.79            |
| S6–F3   | Fungi    | ++             | 4                    | ++            | -            | 271.78                   | 144.84                              | 3.93            |

IAA = indole-3-acetic acid, ACC = 1-aminocyclopropane-1-carboxylate deaminase, TCP = tri-calcium phosphate.

3.2. Quantitative Measurement of Phosphate Solubilization

Phosphorus solubility was significantly influenced by both bacterial and fungal isolates (Table 2). Overall, solubilization of tri-calcium phosphate (TCP) was higher as compared to rock phosphate (RP). Out of seven, only four bacterial isolates were found capable of solubilizing RP whereas all fungal isolates solubilized P, regardless of P sources. Spectrophotometric quantification of growth media showed that maximum RP solubilization was done by S1–2 bacterial isolate and RP solubilization varied between 0–156.52 µg P mL$^{-1}$ (Table 2). While TCP solubilization varied from 33 to 273 µg P mL$^{-1}$ among the different bacterial isolates, maximum solubilization was done by S5–imp bacteria.

In comparison, all fungal isolates solubilized RP and maximum solubilization was done by the S6–F1 isolate (693.16 µg P mL$^{-1}$) isolate (Table 2). Similarly, fungal isolates solubilized TCP and the range varied between 271 to 394 µg P mL$^{-1}$, where maximum solubilization was recorded by the S6–F1 isolate.

Media pH was influenced by the application of microbes in pikoviskaya (PKV) broth amended with TCP or RP (Table 2). It was observed that after seven days of incubation, the pH of the culture supernatant was significantly decreased from the initial pH of 7.0, among bacterial isolates. Bacteria isolate S5–4 lowered the pH to 3.44 while fungal isolate S6–F1 lowered the pH from 7 to 2.79 (Table 2).

3.3. IAA Production and ACC Deaminase Activity

An indole-3-acetic acid (IAA) test was performed to check the isolates for IAA production. All the bacterial and fungal isolates showed positive results for IAA production through HPLC (Table 2, Figure 1).

Isolated strains of bacteria and fungus were examined for ACC deaminase activity. All the bacterial isolates were positive and fungal isolates were negative for ACC deaminase activity by producing turbidity through use of ACC as a nitrogen source (Table 2).
TCP and the range varied between 271 to 394 µg P mL\(^{-1}\), where maximum solubilization was recorded by the S6–F1 isolate. Media pH was influenced by the application of microbes in pikoviskaya (PKV) broth amended with TCP or RP (Table 2). It was observed that after seven days of incubation, the pH of the culture supernatant was significantly decreased from the initial pH of 7.0, among bacterial isolates. Bacteria isolate S 5-4 lowered the pH to 3.44 while fungal isolate S6–F1 lowered the pH from 7 to 2.79 (Table 2).

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An indole-3-acetic acid (IAA) test was performed to check the isolates for IAA production. All the bacterial and fungal isolates showed positive results for IAA production through HPLC (Table 2, Figure 1).

![Figure 1. Indole-3-acetic acid (IAA) production of isolated bacterial and fungal strains.](image)

### 3.4. Gene Sequencing of Bacterial and Fungal Isolates

#### 3.4.1. 16S rRNA Gene Sequencing of Bacterial Isolates

On the basis of phosphate solubility, four bacterial isolates (i.e., S1–2, S2–1, S5–3, S5–imp) were selected and sequenced based on 16S rRNA gene using fD1 and rD1 primers (Table 3, Figure 1). The phosphate solubilizer strain S1–2 was identified as *Staphylococcus* sp. (KY552949) having 94% similarity with the reported gene sequence. S2–1 showed 99% similarity with FJ188300 *Bacillus firmus* strain UST981101-0006 hence identified as *Bacillus*. S5–3 sequence showed 99% similarity with MF078473 *Bacillus Safensis* strain HR-2 and was identified as *Bacillus*. While, S5–imp sequence was 94% similarity with KC813166 *Bacillus licheniformis* strain A2 thus identified as *Bacillus* (Table 3, Figure 2).

| Microbial Isolate Code | Sequence Length (bp) | Sequence Identity % | Nearest Homologue Sequence | Strain Identified                  |
|------------------------|----------------------|---------------------|---------------------------|-----------------------------------|
| **Bacterial Isolate**  |                      |                     |                           |                                   |
| S1–2                   | 1437                 | 94                  | KY552949                  | *Staphylococcus* sp.              |
| S2–1                   | 1527                 | 99                  | FJ188300                  | *Bacillus firmus*                 |
| S5–3                   | 1546                 | 99                  | MF078473                  | *Bacillus safensis*               |
| S5–imp                 | 1215                 | 94                  | KC813166                  | *Bacillus licheniformis*          |
| **Fungal Isolate**     |                      |                     |                           |                                   |
| S6–F1                  | 554                  | 99                  | KJ653467                  | *Penicillium* sp.                 |
| S6–F2                  | 559                  | 99                  | KX219608                  | *Penicillium oxalicum*            |
| S3–F1                  | 590                  | 99                  | KX400571                  | *Penicillium oxalicum*            |
| S4–F1                  | 557                  | 99                  | JQ647900                  |                                   |
Figure 2. Phylogenetic tree of bacterial isolates based on 16S rRNA gene sequences showing the evolutionary position of S1-2, S2-1, S5-3 and S5-imp with other related bacterial species sequences which were retrieved from databases. The phylogenetic tree was constructed in Mega 7 using neighbor-joining method with 1000 bootstrap replications. Statistical bootstrap value is given beside the nodes and gene bank accession numbers are mentioned before strain names.
3.4.2. ITS Gene Sequencing of Fungal Isolates

Four isolated fungal strains were sequenced based on ITS gene (Figure 3). The isolate S$_3$–F$_1$ was identified as *Penicillium oxalicum* having 99% similarity with the reported gene sequence (KX400571). S$_4$–F$_1$ showed 99% similarity with HQ443244 *Penicillium oxalicum* strain FFRh5 and was identified as *Penicillium oxalicum*. National Centre for Biotechnology Information (NCBI) blast nucleotide sequence results of fungal isolate S$_6$–F$_1$ showed 99% similarity with XX219608 *Penicillium sp.* Liaoning-Rsf0085 and was identified as *Penicillium sp.* (Table 3, Figure 3). Nucleotide accession numbers of fungal strains S$_3$–F$_1$, S$_4$–F$_1$, S$_6$–F$_1$ and S$_6$–F$_2$ were MK449353, MK449354, MK449355 and MK449356, respectively.

**Figure 3.** Phylogenetic tree of fungal isolates based on ITS rRNA gene sequences showing the evolutionary position of S$_6$–F$_1$, S$_6$–F$_2$, S$_4$–F$_1$ and S$_3$–F$_1$ with other related fungal species sequences which were retrieved from databases. The phylogenetic tree was constructed in Mega 7 using neighbor-joining method with 1000 bootstrap replications. Statistical bootstrap value is given beside the nodes and gene bank accession numbers are mentioned before strain names.
3.5. Effect of Phosphate Solubilizing Microbes on Maize Growth

Based on the best performance, 2 bacteria (Staphylococcus sp. and Bacillus licheniformis) and 2 fungal isolates (Penicillium oxalicum and Penicillium sp.) were selected to test their impact on maize crop growth fertilized with DAP, RP or vermicompost. Overall results showed that application of P fertilizers in combination with PSMs markedly increased root length, root fresh weight, root dry weight, dry biomass, P uptake and post-harvest soil P (Table 4, Figures 4 and 5). Among the fertilizer sources, maximum root length, root fresh weight, root dry weight and dry biomass were observed in vermicompost treatment and treatments varied in the other vermicompost > DAP > RP. However, only fresh root weight differed statistically significant with different fertilizers (Table 4).

Table 4. Effect of PSMs on maize crop under different P fertilizer sources. Similar letters are not statistically significant at (p ≤ 0.05) among the PSMs and Fertilizers. Different letters show the significance of difference among the PSMs and Fertilizers.

| Inoculants | Root Length (cm) | Root Fresh Weight (g) | Root Dry Weight (g) | Dry Biomass (g) |
|------------|------------------|-----------------------|---------------------|----------------|
| Compost    | 34 ± 0.45 i      | 9.8 ± 0.23 def        | 1.75 ± 0.23 cde     | 3.35 ± 0.18 hi |
| Rock Phosphate (RP) | 29 ± 0.43 i | 6.8 ± 0.35 h         | 1.28 ± 0.25 e       | 2.43 ± 0.17 i  |
| DAP        | 32 ± 0.32 i      | 7.2 ± 0.32 gh         | 1.45 ± 0.09 de      | 3.19 ± 0.03 hi |
| Penicillium sp. × compost | 73 ± 0.45 a | 19.6 ± 0.30 a      | 4.76 ± 0.17 a       | 8.90 ± 0.22 a  |
| Penicillium sp. × RP | 60 ± 0.52 b | 11 ± 0.04 cde       | 2.92 ± 0.09 bc      | 6.13 ± 0.39 bcd|
| Penicillium sp. × DAP | 58 ± 1.13 bc | 12 ± 0.39 bcd      | 2.83 ± 0.23 bc      | 7.36 ± 0.49 bc |
| Penicillium oxalicum × compost | 69 ± 0.45 a | 17.89 ± 0.07 a     | 3.56 ± 0.11 ab      | 7.61 ± 0.28 ab |
| Penicillium oxalicum × RP | 56 ± 0.68 bcd | 10.4 ± 0.61 def   | 2.62 ± 0.12 bcd     | 5.1 ± 0.28 cde |
| Penicillium oxalicum × DAP | 59.66 ± 0.68 b | 11.7 ± 1.30 bcd  | 2.4 ± 0.11 bcd      | 6.24 ± 0.22 cde|
| Staphylococcus sp. × compost | 54 ± 0.68 cde | 14.3 ± 0.19 b    | 2.81 ± 0.26 cde     | 6.23 ± 0.06 bcd|
| Staphylococcus sp. × RP | 42.6 ± 0.68 gh | 9.2 ± 0.28 efg   | 1.71 ± 0.17 cde     | 3.90 ± 0.21 gh |
| Staphylococcus sp. × DAP | 50.6 ± 1.57 ef | 8.1 ± 0.10 fg    | 2.14 ± 0.05 cde     | 4.67 ± 0.16 fg |
| Bacillus licheniformis × compost | 53 ± 0.89 de | 13.5 ± 0.30 bc   | 2.20 ± 0.12 cde     | 5.44 ± 0.13 def|
| Bacillus licheniformis × RP | 40.3 ± 1.13 h | 7.9 ± 0.49 efg   | 1.58 ± 0.09 cde     | 3.1 ± 0.20 ghi |
| Bacillus licheniformis × DAP | 46.3 ± 0.68 fg | 8.5 ± 0.22 fgh  | 1.85 ± 0.42 cde     | 4.78 ± 0.31 fg |

Figure 4. Interactive effect of PSMs and P fertilizer sources on P uptake. Similar letters are not statistically significant at (p ≤ 0.05) among the PSMs and fertilizers. Different letters show the significance of difference among the PSMs and Fertilizers. PSM1 = Penicillium sp.; PSM2 = Penicillium oxalicum; PSM3 = Staphylococcus sp.; PSM4 = Bacillus licheniformis. Compost = vermicompost; RP= rock phosphate; DAP= diammonium phosphate.
Interaction of fertilizer sources (vermicompost) and PSMs (*Penicillium* sp.) resulted in an increase of 53% and 52% in root length and root fresh weight as compared to control, respectively. Overall, as compared to control, root length and root fresh weight ranged between 28–50% and 14–52% among different interactions, respectively. Similarly, as compared to control, root dry weight (63%) and shoot dry biomass (62%) significantly (*p* < 0.05) increased when *Penicillium* sp. was applied in combination with vermicompost. Among different interactions, the corresponding increase in root dry weight and dry biomass ranged between 13–62% and 33–60%, respectively.

Phosphorus uptake and post-harvest soil P was also significantly increased by application of PSMs and P sources. Regardless of P sources, maximum P uptake was observed in soil inoculated with *Penicillium* sp. (Figure 4). While among the different P sources, vermicompost resulted in higher P uptake by maize plants, which was followed by RP and DAP. The interactive effect of vermicompost and *Penicillium* sp. increased the P uptake by 83%. Similarly, analysis of post harvested soil showed that plant available P amount was still higher in all fertilizers and PSMs treatments as compared to control (Figure 5). Irrespective of the P sources, maximum soil P was recorded from treatments where seeds were inoculated with *Penicillium* sp. (Figure 4). However, among the applied treatments from different P sources, vermicompost showed maximum P concentration in post-harvest soil, whereas the interactive effect (vermicompost × *Penicillium* sp.) showed 80% higher P concentration in post-harvested soil as compared to control treatment.

4. Discussion

Bacteria and fungi are among the most important microorganisms that contribute to cycling of insoluble organic and inorganic P in soil and considered as one of the most environmentally friendly and economic methods for improving P availability to plants [4]. In current study, halo zones created by phosphate solubilizing bacteria and fungi can be attributed to release of organic acids, polysaccharides and enzymatic activities like phosphatases by these organisms [19]. Pande et al. [20] also reported the formation of halo zone by phosphate solubilizing microbes, namely *Alcaligenes aquatilis*, *Burkholderiacepacia* and *Alcaligenes aquatilis*.
From the results, it was recorded that the pH of Pikovskaya broth medium was reduced to phosphate solubilizing microbes. This decline in pH might be due to the release of organic acids. The organic acids play vital role in solubilization and transformation of tricalcium phosphate into soluble form of monocalcium phosphate. Yin et al. [21] also reported increased solubility of inorganic phosphate by *Penicillium oxalicum* strain due to production of organic acids and media acidification. Similarly, it is also examined *Penicillium oxalicum* for its rock phosphate solubilizing capacities in PKV media amended with different levels of RP and reported the highest P solubilization by this strain in the media.

A vast majority of bacterial isolates are able to produce auxins like indole-3-acetic acid (IAA) as a part of their metabolism which play an important role in plant growth and development. In the current study, isolated bacterial strains were also analyzed for their plant growth promoting abilities through IAA production. Except fungi, all the bacteria isolates were rated positive for production of IAA which implies their added contribution in plant growth and development. It is reported that *Stenotrophomonas* sp. and *S. rhizophila* bacterial strains were capable of IAA production. Similarly, it is also reported that *Staphylococcus* sp. and *Bacillus* sp. were capable of producing IAA and solubilized P [3,9].

Like indole-3-acetic acid, ACC (1-aminocyclopropane-1-carboxylic acid) deaminase activity is also a plant growth promoting trait that helps plants to grow in stress conditions. Khan et al. [22] reported IAA and ACC deaminase induce drought tolerance in wheat and chickpeas grown on dry lands. They highlighted the role of microorganisms to manage abiotic and biotic stress by producing indole-3-acetic acid (IAA) and ACC-deaminase to reduce levels of ethylene in roots. Microorganisms capable of producing ACC deaminase degrade ethylene and use the resultant products as a nitrogen source for their growth [23]. In our study, all six isolated bacteria were marked ACC positive. Khamwan et al. [24] identified *Bacillus*, *Curtobacterium* and *pseudomonas* as endophytic bacteria capable of performing ACC deaminase activity.

On the basis of phosphate solubilization index and quantitative determination of P, four bacterial isolates and four fungal isolates were selected for PCR amplification of 16S rRNA and 18S rRNA, respectively, using forward and reverse primers in a 25 ul reaction. The sequences from Macrogen were contiged using Seqman MEGA 7 software and BLAST online to NCBI. As a result, bacterial isolates were identified as *Staphylococcus* sp. *Bacillus firmus*, *Bacillus safensis*, and *Bacillus licheniformis*, while fungal isolates were identified as *Penicillium* sp. and *Penicillium oxalicum*. The results can be correlated with the previous work of Singh et al. [25] who also identified fungal species as *Penicillium oxalicum* isolated from rock phosphate mines.

Two of each bacterial and fungal strains were tested under a soil-plant environment with different P source inputs. As compared to RP and DAP, inoculation of bacterial and fungal strains with vermicompost resulted in prominent growth in terms of root length, root fresh weight, root dry weight, dry biomass, P concentration in leaves, P uptake and post-harvest soil P status. In fact, PSMs release phosphatase enzymes that help solubilization of P which enhances growth and yield of plants. Study showed better wheat growth (i.e., root length, shoot length, root biomass and shoot biomass) where soil was amended with compost, RP and phosphate solubilizing bacteria. The effect of *Penicillium sp.* was more pronounced on plant growth when combined with compost as compared to other PSMs and P fertilizer treatments, which could be attributed to the potential of these isolates to increase the availability of nutrients such as phosphorus by production of organic acids and lowering the soil pH for nutrient availability [8,26]. It is reported that mineralization of nutrients through inoculation of PSMs could be the major mechanism for increase in plant growth.

Similarly, P uptake increased significantly with the addition of PSMs. The highest increase was observed with application of *Penicillium* sp. in compost. The result could be due to higher P solubilizing capacity of *Penicillium* sp. and improved root growth which
ultimately increased P uptake [27]. Kumar and Singh [28] also found that application of phosphate solubilizing bacteria (Pseudomonas striata) to vermicompost enhanced the availability of P in soil. Nishanth et al. [29] reported that compost inoculated with Aspergillus awamori can significantly enhance P uptake by wheat crop and efficiency was increased up to 78% as compared to DAP. A three-fold increase in wheat P uptake with PGPR inoculation with different P fertilizers was reported. Furthermore, incorporation of organic materials by itself can enhance P availability in the soil solution by decreasing P sorption/fixation through chelation. Thus, application of PSMs and compost might have a synergistic effect on P uptake by plants.

On the other hand, available P in post-harvested soil was higher from the posts amended with compost. The ligand exchange reactions can increase P mobilization through organic and phosphate anions adsorption on Fe and Al sites. The improvements in soil P are attributed to the positive effect of Penicillium sp. which enhanced the solubility and release of phosphorus. Kumar et al. [30] reported improvements in soil P by application of organic manures in combination with phosphate solubilizing microbes. The increase in post-harvest soil P availability with application of compost in combination with Penicillium sp. may be due to the mineralization of both compost and soil organic P, and chelation of P through ligand exchange reactions to reduce P fixation throughout the crop growing stages. Thus, in synergistic relations of Penicillium sp. and compost, Penicillium sp. enhanced the efficiency of compost by improving the P availability and compost provided optimum conditions for microbial growth and multiplication, resulting in overall better plant growth.

5. Conclusions

Current research revealed that the Hazara phosphate mines are well blessed with efficient phosphate solubilizing microbes, having remarkable potential to solubilize rock phosphate as well as mineralize fixed phosphorus pools of the soil. Improvement in phosphorus bioavailability for maize plant uptake by Penicillium sp. inoculation has shown that a specific strain can be used as bio-inoculant for mineralization of soil indigenous phosphates which have previously been fixed after continuous and indiscriminate application of phosphate fertilizers. Use of promising bio-inoculants along with suitable organic fertilizer can decrease the cost of production by mineralizing soil organic phosphorus for better crop productivity. However, the role of different sources of organic fertilizers inoculated with PSMs under different cropping systems needs further investigations.

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