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ORIGINAL PAPER

Phosphate solubilizing epilithic and endolithic bacteria isolated from clastic sedimentary rocks, Murree lower Himalaya, Pakistan

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Abstract

Rock microbes are capable to solubilize phosphate present in the rocks.. In this study, we focused on the isolation of phosphate solubilizing bacteria from rocks of Murree, Pakistan. Both endolithic and epilithic bacteria were screened for phosphate solubilization. Three bacterial strains were selected based on halozone formation inNational Botanical Research Institute for phosphate) medium supplemented with TCP (tribasic calcium phosphate). The solubilization index for these bacteria was recorded as 4.29, 4.03 and 3.99. The pH of the medium dropped from 7.0 to 4.0 after 5 days with continuous shaking at 150 rpm, which facilitate the phosphate solubilization. The strains P26, P4 and N27 were identified as *Pseudomonas putida* strain (KT004381), *Pseudomonas grimontii* (KT223621) and *Alcaligenes faecalis* (KT004385). Strain P26 showed maximum phosphate solubilization (367.54 µg/ml), while P4 and N27 showed 321.88 and 291.36 µg/ml after 3 days of incubation. Such inorganic phosphate solubilization could be attributed to the organic acids production by bacteria. The presence of organic acids is determined by high-performance liquid chromatography. Three different types of acids, gluconic, oxalic and malic acid were the dominant acids found in the culture medium. It may be assumed that these bacteria can play a role in weathering of rocks as well. PSB is likely to serve as an efficient biofertilizer, especially in areas deficient in P to increase the overall performance of crops.

Keywords Endolithic · Epilithic · Phosphate solubilizing bacteria · Organic acids · Biofertilizer

Introduction

Phosphorus is the second most important macronutrient for plant growth after nitrogen in the terrestrial environment (Walker and Syers 1976; Vitousek and Howarth 1991; Ahmad et al. 2005; Farajzadeh et al. 2012; Alori et al. 2017; Fatima et al, 2021). Global metagenomics studies of various terrestrial ecosystems revealed that the terrestrial plant's response to phosphorus addition was not different from those to nitrogen addition (Elser et al. 2007; He et al. 2010; Zhang et al. 2018; Liang et al. 2020). Phosphorus is found in almost all rocks and regarding availability to plants it is one of the less available elements in the lithosphere (Jones and Oburger 2011; Alloway 2013; Etesami and Jeong 2021), therefore considered a limiting nutrient in the soils. Phosphates are the fully oxidized forms of phosphorus, but it forms insoluble complexes with aluminum, calcium and iron, thereby generating insoluble phosphate salts with low solubility, affecting the productivity of ecosystems (Etesami and Jeong 2021). For maximum plant productivity, a large quantity of soluble P in the form of chemical fertilizers is applied to soils.

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However, this soluble P is easily precipitated into insoluble forms like $FePO_4$, $CaHPO_4$, $AlPO_4$ and $Ca_3(PO_4)_2$ which are not easily taken up by plants, what leads to soil pollution or can be washed away to fresh water and groundwater (Omar 1998; Shigaki et al. 2006; Hazra and Das 2014; Khan et al. 2018; Zhang et al. 2022). To solve this problem, it is necessary to develop a technology that should be eco-friendly and economical (Vassilev and Vassileva 2003).

Phosphate solubilizing microbes (PSMs) are a group of beneficial microorganisms capable of hydrolyzing organic and inorganic insoluble phosphorus compounds to soluble form that can easily be assimilated by plants. PSM provides an eco-friendly and economically sound approach to overcome the P scarcity and its subsequent uptake by plants. Though PSMs have been a subject of research for decades, manipulation of PSMs for making use of increasing fixed P in the soil and improving crop production at the field level has not yet been adequately commercialized. PSMs have the ability to solubilize insoluble phosphate. They not only provide phosphorus but can also accelerate the accessibility of trace elements (Mittal et al. 2008; Zhang et al. 2022), facilitate plant growth by the fixation of atmospheric nitrogen (Dobbelaere et al. 2002; Sahin et al. 2004; Khan et al. 2018), produce plant growth hormones like auxins (Jeon et al. 2003; Egamberdiyeva 2005), cytokines (Salamone et al. 2001), and gibberellins (Gutierrez-Manero et al. 2001), enzymes and fungicidal compounds such as protease, chitinase and cellulase (Dey et al. 2004; Lucy et al. 2004; Hamdali et al. 2008). Therefore, it is believed that PSMs greatly affect plant performance by the production of the substances which promote plant growth (Hameeda et al. 2006a). Due to their ability to solubilize inorganic phosphorus pools, PSMs are widely used as inoculants to increase crop yield (Chen et al. 2008; Khalid et al. 2004; Hameeda et al. 2006b). Using PSMs as inoculants for phosphorus availability and plant growth promotion have been assessed in several studies in field conditions and greenhouse (Reyes et al. 2002; Zaidi et al. 2003).

Different kinds of PSB have been characterized and reported for promoting plant growth and increasing the availability of phosphorus (Khan et al. 2010; Zaidi et al. 2009; Rodríguez and Fraga 1999; Harris et al. 2006). Different mechanisms are used by PSB to bring about the solubilization of phosphates from insoluble forms into soluble, but the most efficient mechanism used by these bacteria is the production of microbial metabolites such as organic acids (Lin et al. 2006). These organic acids contain carboxyl and hydroxyl groups which are responsible for the conversion of phosphates into soluble forms (Chen et al. 2006). The organic acids produced by these microorganisms are of low molecular weight and the concentration of acids varies from 1 to 50 Mm (Strobel 2001). Such acid-producing microbes are also colonized in rocks (Sajjad et al. 2019a). The rocks in lower Himalayas, Pakistan, provide a suitable environment for epilithic and endolithic bacteria (Ali et al. 2020; Khan et al. 2021) that are active in the weathering of rocks. Therefore, this study was aimed to isolate and characterize PSB from rocks in the lower Himalayas. In addition, the phosphate solubilizing potential of the isolates was evaluated with the view of using them as a source of biofertilizer to boost soil fertility.

Materials and methods

Isolation of phosphorus solubilizing bacteria

The rocks used for PSB isolation were collected from the lower Himalayas in Ayubia, Abbottabad, Pakistan, (Elevation 2600 m), (34°4′20"N, 73°23′55"E). Both epilithic and endolithic strains isolated from these rocks were used for phosphate solubilizing activity in NBRIP (National Botanical Research Institute for phosphate) medium containing 10 g glucose, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 5 g $MgCl_2.6H_2O$, 0.1 g $(NH_4)_2SO_4$ in 1 L distilled water. For the selective screening of PSB, 5 g of tribasic calcium phosphate (TCP) was used in which an inorganic source of phosphorus is added as a sole source of phosphate (Nautiyal 1999). For epilithic bacteria, the rock surface was swabbed and grown on a medium, while for endolithic bacteria, rocks were broken down and swabbed the inner surface and streaked on a medium. All the strains were point inoculated on these plates and were incubated for 5 days at 30 °C. Phosphate solubilizing colonies were selected based on the formation of clear zones.

Solubilization index

The solubilization index was determined by measuring colony diameter and clear zone diameter, using the formula used by Edi-premono et al. (1996).

$$SI = \text{Colony diameter} + \text{Halozone diameter}/\text{Colony diameter}.$$

Bacteria that formed clear zones around the colonies could solubilize inorganic phosphate and were transferred to a liquid NBRIP medium. All assays were performed in replicates.

Quantitative analysis of phosphate solubilization

For the quantitative analysis of TCP, all the PSB strains were grown in a liquid NBRIP medium. 0.1 ml of bacterial were added to liquid NBRIP medium and the flasks were incubated for 7 days at 27 °C in a shaker incubator at 180 rpm. After incubation, the cultures were centrifuged at 13,000 rpm for 10 min. The supernatant was collected from

the samples and available phosphate was estimated by the molybdenum blue method by using a spectrophotometer at the wavelength of 600 nm (OD_{600nm}). The absorbance of the samples was measured on the same wavelength using standard curve concentrations of converted *P* were expressed as µg ml^{-1} (Fernández et al. 2007).

Effect of pH and temperature on phosphate solubilization

To study the effect of pH and different temperatures on phosphate solubilization, the isolates were incubated at various pH (4, 5, 6, 7, 8 and 9) and temperatures (25, 30, 35, 40, 45 °C). The isolates were grown in a liquid NBRIP medium for 3 days and solubilization was observed based on the phospho-molybdate blue color method.

Identification of PSB strains

DNA extraction

PSB strains were grown in 10 ml nutrient broth for 24 h at 30 °C. About 1 ml of each culture was transferred to microtubes and centrifuged at 13,000 rpm for 5 min and the pellet was collected for total DNA extraction using the CTAB method (Sajjad et al. 2016). The extracted DNA was confirmed and visualized using an Ultraviolet–visible spectrometer on gel electrophoresis containing ethidium bromide and 1% agarose gel.

Sequencing and identification

Polymerase chain reaction (PCR) was performed using universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR products were then sent to Macrogen (Korea) for sequencing. Once the sequencing was done, the phylogenetic tree was constructed using the online BLAST program comparing the related sequences with the available known sequences in the NCBI data bank (http://www.ncbi.nlm.nih.gov/BLAST).

Phylogenetic analysis

Related sequences were obtained from NCBI and aligned with the muscle software. The pairwise aligned sequences were processed for the construction of a phylogenetic tree. The phylogenetic tree was inferred using MEGA X software. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.24778990 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 893 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

Analysis of pH change and production of organic acids

For the analysis of pH change, PSB solubilizing bacteria were grown in a liquid medium and were incubated for 7 days at 30 °C. The cultures were centrifuged after incubation, and pH of the medium was recorded for organic acid production by PSB strains. The supernatant was collected from the liquid medium and was centrifuged at 13,000 rpm for 15 min. Then the samples were filtered through 0.2 um filters and 20 ul of filtrates were subjected to High-performance liquid chromatography (HPLC).

Results and discussion

Identification of PSB strains

Rocks harbor biologically active microbes that carried out several biogeochemical cycling (Sajjad et al. 2022). The rocks in lower Himalayas Pakistan provide a suitable environment for epilithic and endolithic bacteria (Ali et al. 2020). In this study, bacterial strains obtained from rocks were studied. All the bacterial isolates were tested for phosphate solubilization. Among these isolates, only three strains (two epilithic and one endolithic) developed a clear zone around the colonies after 7 days of incubation that shows phosphate solubilization. Epilithic Strain P4 was identified as *Pseudomonas grimontii* P4 (KT223621) and another epilithic strain P26 was identified as *Pseudomonas putida* (KT004381). Similarly, the endolithic strain N27 showed the nearest proximity with *Alcaligenes* species and was identified as *Alcaligenes faecalis* N27 (KT004385) (Fig. 1).

Calcium phosphate ($Ca_3(PO_4)_2$) was used as a source of phosphate and dissolved by the isolated bacterial strains. Similar findings were also found by Antoun et al. (2009), which showed that only those bacteria have the potential to form halozones in a medium which composed of Ca-P as a source of phosphate. The formation of halozones is because of glucose transformation into an organic acid (Widawati 2011). In the isolation of PSB from the sea, glucose was

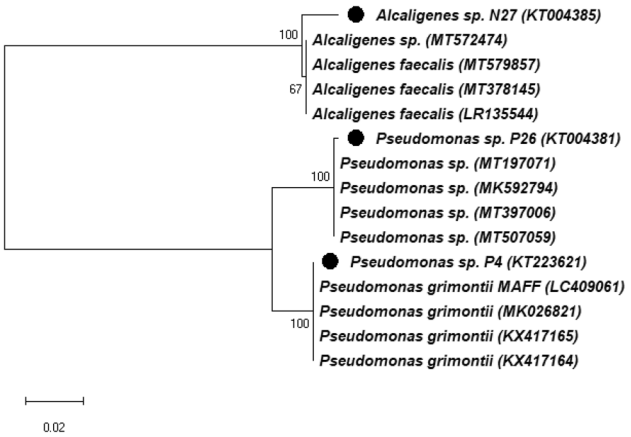

Fig. 1 Phylogenetic tree of the isolates based on 16S rRNA sequences showing the positions of *Pseudomonas grimontii*, *Pseudomonas putida* and *Alcaligenes faecalis* strains with respect to the closest species. The accession numbers are of the isolated species are also given in parenthesis

utilized as the carbon source to dissolve the bound phosphor indicated by halozones (De Souza et al. 2000). Seshadri et al. (2002) and De Souza et al. (2000) reported that bacteria with phosphate solubilization potential from coastal, offshore, mangrove and seawater could dissolve phosphate from zinc phosphate (30%), calcium phosphate (19%) and calcium triphosphate (18%).

The highest solubilization efficiency was observed in two isolates P26 and P4, there was a slight difference in the solubilization rate of all three strains. The size of the halozones determines the capability level of bacteria to dissolve the bonded phosphate (Rachmiati 1995). The larger the halozones developed, the more likely the isolates are to dissolve the bound P shown in (Fig. 2).

The capability of phosphate solubilization by the isolated endolithic and epilithic bacteria to quantitatively solubilize phosphate and pH of the broth medium with phosphate source ($Ca_3(PO_4)_2$) after incubation of 7 days was measured by spectrophotometer, which showed that the three isolates P26 (367.54 µg ml^{-1}), P4 (321.88 µg ml^{-1}) and N27 (291.36 µg ml^{-1}) have the potential to solubilize the inorganic phosphate of $Ca_3(PO_4)_2$ in the liquid NBRIP medium. When the phosphate was solubilized in the medium, the pH of the medium was dropped. The solubilization of phosphate alters the pH with the production of organic compounds released to the medium, redox reactions and organic ligand competitors (Cunningham and Kuiack 1992). Jeon et al. (2003) reported that *Pseudomonas fluorescens* solubilizes $Ca_3(PO_4)_2$ after incubation for 5 days and pH drops to 4.4. The acidity of the liquid medium is the key mechanism for the dissolution of the minerals (Sajjad et al. 2020). Therefore, the acidity of the liquid medium supports phosphate solubilization (Perez et al. 2007). Whitlaw et al. (1999) also reported that the concentration of phosphate solubilized in liquid medium is in line with the acidity and concentration of amino acid and also pH shift. According to Rao (1982), the process of inorganic phosphate solubilization, including $Ca_3(PO4)_2$, includes pH changes caused by the synthesis of organic acids such as acetate, citrate, and oxalate. Ramachandran et al. (2007) stated that, since the bacteria can discharge inorganic phosphate from $Ca_3(PO_4)_2$ in liquid culture, the bacteria have the capacity to dissolve the bound P and so make it accessible to the plants.

Solubilization index

The solubilization index was measured by the formation of halozones around the colonies growing on a solid NBRIP medium. The formation of halozones was the indication of these strains to solubilizing insoluble TCP. The varying diameters of the halozones showed that TCP was solubilized to different degrees by different strains. All the indices of solubilization are shown in Table 1 grown on NBRIP solid medium. In the first 24 h, rapid halozones were observed, but with the passage of time and with the increase of colony diameter, the zones expanded. It was due to assimilation and then deficiency of available phosphate in the medium. Our results showed that P26 was the most efficient in solubilizing inorganic phosphate on NBRIP solid medium with a solubilization index of 4.29, followed by P4 and N27 with solubilization indices of 4.03 and 3.99 respectively (Table 1).

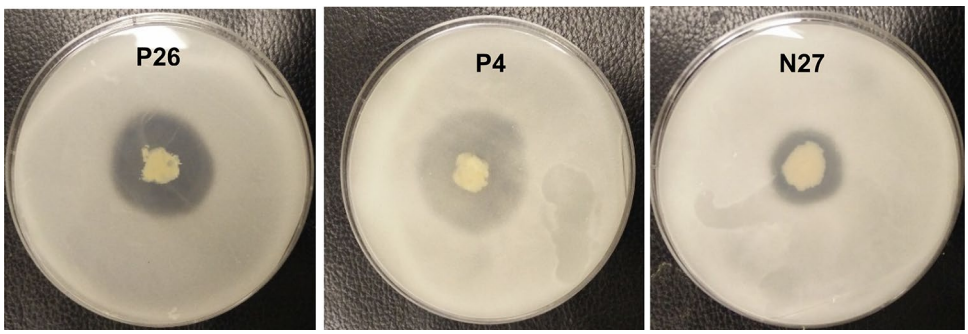

Fig. 2 Zone of hydrolysis by different strains

Table 1 Solubilization index

Strains	Colony diameter (cm)	Zone diameter (cm)	SI index
N27	2.8	3.2	3.9
P26	2.1	4.6	4.29
P4	1.2	3.4	4.03

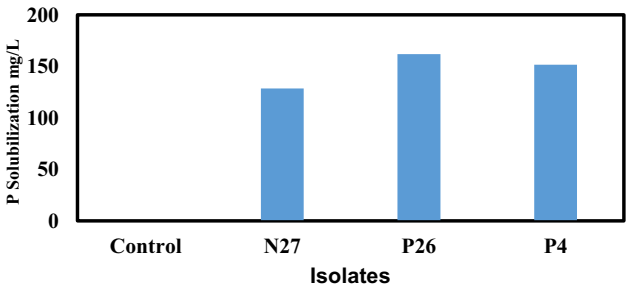

Fig. 3 Orthophosphate solubilization from inorganic and insoluble tricalcium phosphate by the isolated strains

Quantitative analysis of phosphate solubilization

In this study pH of the medium containing TCP was observed to decrease during the bacterial incubation. The initial pH of the medium was set at 7. The results showed pH of the medium decreased gradually during the first few days. The pH of the culture medium decreased to 4 in the beginning and remained almost constant afterward. All the phosphate solubilizing strains began to grow exponentially when grown in an NBRIP medium containing TCP as the sole phosphate source. The pH of the medium also started to decrease from the initial pH of 7 and continued to decrease till the stationary phase.

Importantly, the acidification is accredited to the consumption of glucose present in the medium which results in the production of organic acids. Previous studies also showed similar results (Trivedi and Sa 2008; Rodriguez et al. 2004). Reduction in the quantity of soluble phosphate is the result of auto-consumption by bacterial cultures growing in a TCP medium. (Rodriguez et al. 2000). This element liberated is used to estimate the phosphorus released, which was either assimilated by bacteria to form biomass or dissolved as orthophosphate in the supernatant (Fig. 3).

In the current study, the maximum released soluble orthophosphate was recorded for P26 (161.70 mg/l) after 3 days of growth. This was followed by P4 (151.60) and N27 (128.37) after the same time of incubation. No significant change was observed in pH and phosphate concentration in uninoculated controls. A clear relationship can be established between the growth of bacteria, solubilization of orthophosphate from TCP and acidification of supernatant.

Effect of pH and temperature of phosphate solubilization

The isolates showed good growth between 20 and 45 °C, which indicates that they can solubilize phosphate between these ranges. Isolates *Alcaligenes faecalis* N27, *Psuedomonas* sp. P26 and *Pseudomonas grimontii* P4 showed good phosphate solubilizing activity at 30–35 °C. While the optimum pH range was from 4 to 9, the best solubilization activity was shown at pH 7 (Fig. 4).

Our results show that the optimum temperature for phosphate solubilization by the bacteria isolates was 30 °C. Similarly, (Johri et al. 1999; Fasim et al. 2002; Rosado et al. 1998 and Kim et al. 1997) have shown that 30 °C is the best temperature for solubilization. Few researchers have reported 28 °C as the optimum temperature for phosphate solubilization (Seshadre et al. 2002; Kang 2002), whereas Sayer and Gadd (1998) and Gharieb et al. (1998) reported 25 °C as the optimum temperature for phosphate solubilization. Solubilization at extreme temperatures has been reported by some

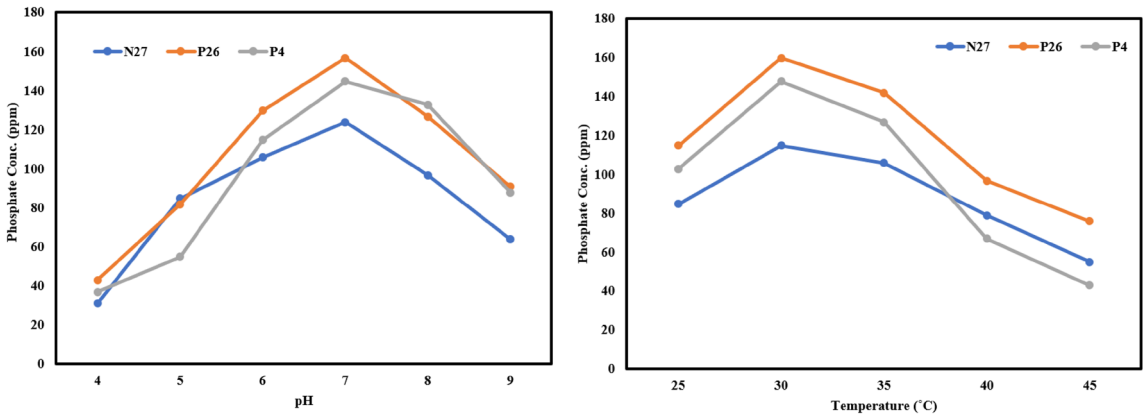

Fig. 4 Phosphate solubilization by the study isolates and effect of pH and temperature on phosphate solubilization

scientists. Solubilization of phosphate has been reported at temperatures as low as 1 °C (Johri et al. 1999) and as high as 45 °C (Nautiyal et al. 2000; Nahas 1996) is also reported.

Phosphate solubilization is the result of acid production by bacteria which lowers the pH; however, there may also be other mechanisms involved (Fasim et al. 2002; Nguyen et al. 1992; Sayer and Gadd 1998). Organic acids production results in the solubilization of phosphate in many cases. At pH 12, metabolites other than organic acids like siderophores are responsible for phosphate solubilization (Nautiyal et al. 2000; Nahas 1996). Phosphate solubilization is attributed to a multitude of factors which include microorganisms, pH decrease and insoluble phosphate (Nahas 1996). It is clear from these studies that bacteria have the ability to adapt to the environment the surroundings and environmental conditions are linked with bacterial metabolic activities.

Organic acids production

Production of organic acids by microbes using precursors in the medium through biosynthesis is the highly reported mechanism to solubilize phosphates (Rodríguez and Fraga 1999). Production of organic acids by PSB strains isolated from Murree rocks, in NBPIR broth, was investigated. Culture filtrates showed the presence of different organic acids confirmed through HPLC chromatography analysis which convened the capacity of these bacteria to solubilize inorganic phosphate from TCP. Phosphates are either dissolved by the organic acids produced through anion exchange or they may chelate Fe, Al or Ca associated with insoluble phosphates (Gyaneshwar et al. 2002). Different types of acids were produced by PSB strains. The acids dominantly produced by our isolates were malic, oxalic and gluconic acids. Past studies on the production of organic acids by microorganisms (Illmer and Schinner 1992; Whitelaw et al. 1999) showed that in their experiments gluconic acid was not produced by the bacterial strains. Our findings two strains did not produce gluconic acid while one strain was able to produce it (Fig. 5).

Alam et al. (2002) reported that oxalic acid is the most dominant acid produced, the same is the case in our study. One of the most reported mechanisms by which microorganisms solubilize phosphate and liberate phosphorus from insoluble phosphate minerals is the production of gluconic acid through the activity of periplasmic cell-membrane bound NADP-dependent glucose dehydrogenase (Goldstein 1995). The acidification of periplasmic space which results in solubilization of phosphate is attributed to the direct oxidation of glucose or other aldose with the help of quinoproteins and glucose dehydrogenase (PQQGDH). When gluconic acid is produced by the conversion of glucose, a transmembrane proton is generated which is used for transport functions and bioenergetics of the membrane and thus the gluconic acid protons become available for phosphate solubilization (Liu et al. 1992).

Correlation with other studied parameters

The results in our study show a strong negative correlation ($r = 0.994$; $p < 0.01$) between the released soluble phosphorus and culture pH which proves that the inorganic phosphates are dissolved and solubilized by the decrease of pH for previously studied bacterial strains (Hwangbo et al. 2003; Chen et al. 2006). This confirms the hypothesis of the involvement of organic acids in solubilizing the insoluble phosphate into a soluble form (Vazquez et al. 2000; Hwangbo et al. 2003; Perez et al. 2007; Chen et al. 2006; Trivedi and Sa 2008).

The decrease in pH of the culture medium and organic acid production by microorganisms is the reason for the solubilization of orthophosphate from TCP (Sajjad et al. 2018; 2019b; Puente et al. 2004; Carrillo et al. 2002; Illmer and Schinner 1995). A decrease in pH is the main mechanism for inorganic phosphate solubilization (Rodriguez and Fraga 1999; Illmer and Schinner 1992). Another mechanism for phosphate solubilization is the production of acid chelates. In the presence of Ca^{2+}, these chelates alter the solubility of the product thereby releasing the insoluble phosphate (Puente et al. 2004; Vazquez et al. 2000; Nautiyal et al. 2000). The correlation between organic acids and pH was found to be highly negative and had a positive correlation for organic acids and released orthophosphates. Organic acids are perhaps the main reason for the solubilization of phosphate, same results were provided by Whitelaw et al. (1999) and Nahas (1996), proving that in many cases the important and key mechanism for phosphate solubilization was acid production. We can conclude that the production of organic acids in large quantities results in the acidification of bacterial cells and the surrounding environment which in turn results in the release of orthophosphate from inorganic phosphate minerals by proton substitution for Ca^{2+}. Thus it is a fact that our strains produced organic acids like gluconic, oxalic and malic acid, which is a property of PSM members of the family *Enterobacteriaceae* (Hameeda et al. 2006a, b; Buch et al. 2008; Lin et al. 2006).

It is the first report on bacteria isolated from the rocks of Murree hills in lower Himalaya Pakistan which exhibits a high capability of solubilizing phosphate in an NBPIR medium in which TCP was used as the sole phosphorus source. A sharp decrease in pH was recorded in our study along with the solubilization of TCP. This indicated the production of organic acids from the metabolic reactions of the microorganisms which resulted in the solubilization of orthophosphate (Farhat et al. 2009; Hwangbo et al. 2003). The hypothesis that the solubilization of $Ca_3(PO_4)_2$ and rock phosphate is caused by the secretion of organic acids

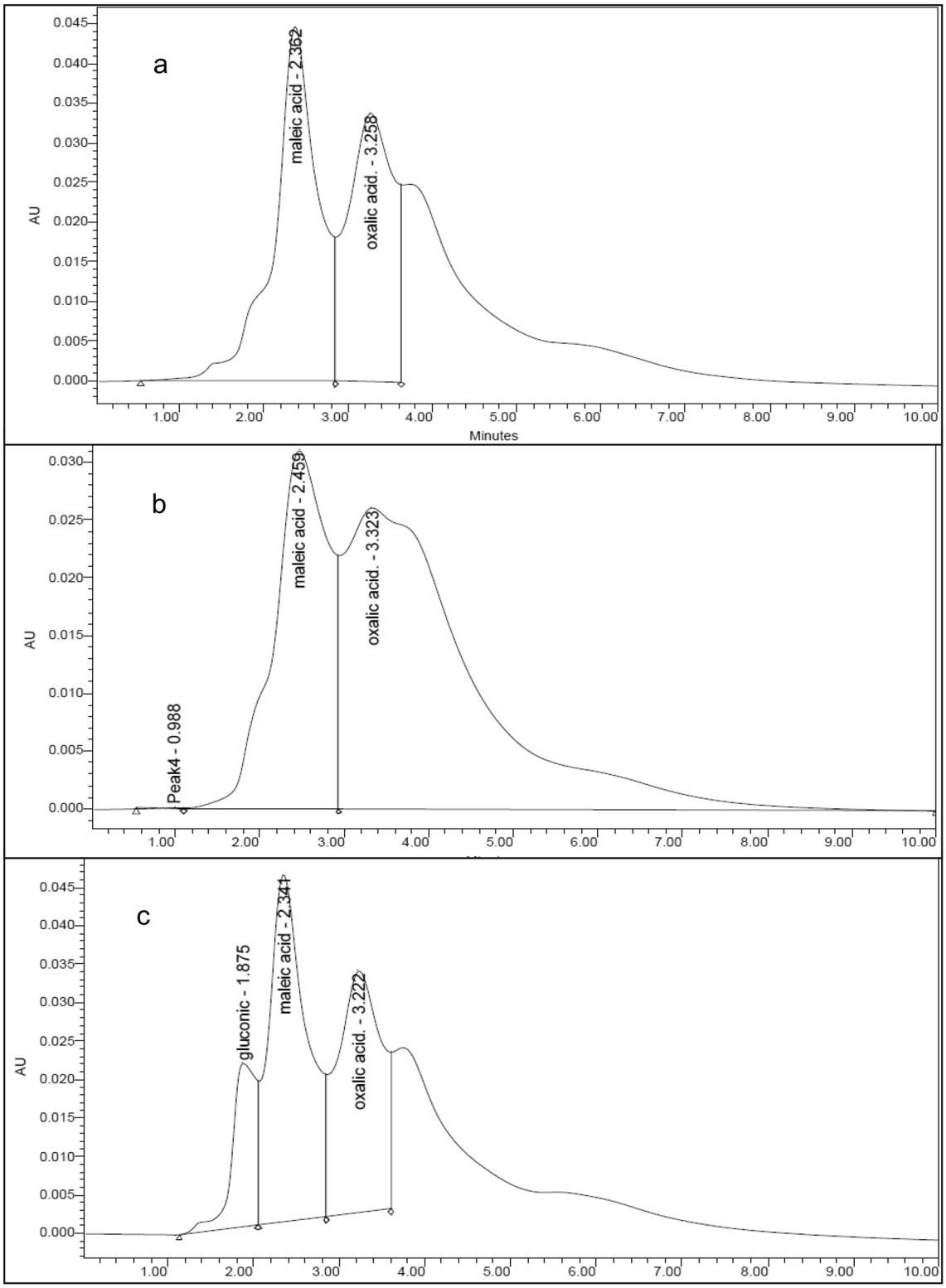

Fig. 5 HPLC identification of organic acids produced by three strains *Pseudomonas grimontii* (**a**), *Pseudomonas putida* (**b**) and *Alcaligenes faecalis* (**c**)

was confirmed by HPLC chromatography analysis which revealed that organic acids are produced during bacterial growth in the NBPIR medium. These results along with the formation of halozones indicate that strains isolated from these rocks have the ability to solubilize phosphorus from inorganic rock minerals of phosphate. These PSB strains can be used for crop yield as they promote plant growth by providing them with soluble phosphorus and in developing inoculants that fulfill the nutritional requirement of the plants. These types of bacteria are usually environment-friendly. All the nucleotide sequences and data are available in the GeneBank database with their accession numbers.

Conclusion

It is concluded from this study that these endolithic and epilithic bacteria having the ability of solubilizing rock phosphates can be used to improve soil quality and phosphate availability. The organic acids produced by these PSB strains are responsible for phosphate solubilization and also play an important role in phosphate rock weathering The endolithic and epilithic PSB can be used as bio-inoculants to increase the bioavailability of phosphate present in the soil as phosphate is mostly present in insoluble form making complexes with other elements. Applying these bacteria will help to minimize the use of synthetic fertilizers which will reduce environmental pollution and play role in sustainable agriculture.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by IK and SZ. The first draft of the manuscript was written by IK, SZ, MR, WS and SZ. FH revised and edited the final manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The current data is PhD research work. The authors declare that they have no conflict of interest.

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