Original Article
ISSN (Online): 2350-0530
ISSN (Print): 2394-3629

International Journal of Research - GRANTHAALAYAH
April 2024 12(4), 162–173

ISOLATION AND IDENTIFICATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM WASTE DUMPING GROUND IN MUMBAI

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Received 03 April 2024
Accepted 05 May 2024
Published 18 May 2024

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DOI
10.29121/granthaalayah.v12.i4.2024.5635

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

ABSTRACT

Phosphate solubilizing bacteria can solubilize insoluble phosphate complexes and convert them into available forms that can be used by plants for better growth. Phosphorus in chemical fertilizers gets fixed in the soil and becomes unavailable for plant growth. It is important to find an alternative inexpensive and sustainable technology that could provide sufficient Phosphorus nutrition to plants. An efficient Phosphate solubilizing bacteria was isolated based on its solubilization zone on Pikovskaya's agar. The amount of Phosphate solubilized by the bacterial isolate was 490.0 ug/ml which was significantly higher as compared to control S. aureus which was 131.0 ug/ml. The release of soluble P significantly correlated with a drop in pH from 7.00 to 3.85 indicating the acid production mechanism of Phosphate solubilization. The isolated bacterial strain could also mineralize organic sources of phosphate. It also showed potential to solubilize phosphate under stress conditions such as heavy metals and salt. The Phosphate solubilizing bacteria was identified by MALDI-TOF sequencing and was shown to belong to the genus Serratia. Therefore, the isolated bacterial strain shows a good potential to be used as a biofertilizer and provide phosphate nutrition to the plants sustainably.

Keywords: Phosphate Solubilization, Serratia, Phosphate Solubilization Index, Heavy Metals, pH, Salt, MALDI-TOF

1. INTRODUCTION

Soil is one of the most important resources which comprises abundant microorganisms. These microorganisms play an important role in various plant growth-promoting activities and cycling of nutrients Singh et al. (2015). Phosphorus (P) is the second major plant growth-limiting nutrient after Nitrogen (N) that affects plant growth and productivity. Although Phosphorus is abundant in soils, its update is limited as it readily forms insoluble complexes with Ca, Al, Mg, Mn, and Fe Wan et al. (2020), Ben et al. (2009). To Overcome Phosphorus deficiency, many chemical

fertilizers used in agricultural sectors pose a threat to the environment causing soil pollution and polluting runoff water bodies. Moreover, the applied phosphate chemical fertilizers are easily fixed into insoluble forms such as $Ca_3(PO_4)_2$, $FePO_4$, $AlPO_4$ etc. Patel et al. (2008), Park et al. (2009).

Some microorganisms present in the soil are capable of solubilizing phosphate which acts as a natural biofertilizer leading to plant growth and development Park et al. (2009), Dhurve et al. (2017) Several mechanisms such as the production of organic acids, secretion of siderophores, production of CO_2, nitrogen assimilation has been suggested on phosphate solubilization by bacteria Xie et al. (2021). Production of organic acids by bacteria converts insoluble phosphates to soluble forms by chelating the cations bound to phosphate Mardad et al. (2013), Chen et al. (2006). Several phosphorus solubilizing bacteria belonging to different species such as *Pseudomonas, Serratia, Burkholderia, Pantoea, Bacillus, Enterobacter, Alcaligenes, and Citrobacter* have been identified and studied for their phosphate solubilization potential Henri et al. (2008), Pérez et al. (2007), Banerjee et al. (2010), Gyaneshwar et al. (1999), Pande et al. (2017), Patel et al. (2008). The diversity of these soil microbes varies depending on soil conditions pH, temperature, contamination due to heavy metals, and nutritional content of the soil Ndung'u-Magiroi et al. (2012). The application of phosphate-solubilizing microbes can help improve soil quality and can be a great method of sustainable agriculture Wan et al. (2020).

Phosphate solubilizing microbes have been isolated from different soils including metal-contaminated areas, solid waste compost, acidic soils, saltern sediments, in addition to rhizosphere soil Xie et al. (2021). However, many of these microorganisms are not able to survive in new environments when used as biofertilizers mainly because of the difference in soil characteristics from where the bacteria were isolated Susilowati et al. (2019). Bacterial isolates from harsh or stressful environments such as waste dumping sites can prove to be better adapted to stress conditions. Therefore, the isolation of bacteria from stressful environments can prove useful as it increases the chances of bacterial survival in any given soil environment, thus making it a good candidate as a biofertilizer. Bacteria from soils that are drought-prone and are subject to changing PH and temperature should exhibit survival strategies in such soil to sustain their growth, which also forms the basis of the "stress physiology paradigm" Pérez et al. (2007). It was found that phosphate-solubilizing microorganisms do not perform consistently as they show poor adaptability to changes in soil and climatic conditions Dhurve et al. (2017). No study has been reported regarding the ecology of phosphate solubilizing bacteria of Mumbai and its polluted sites. The objective of the current study was to isolate effective phosphate-solubilizing bacteria in polluted sites of the Mumbai region and the scope of their use as potential biofertilizers.

2. MATERIALS AND METHODS

2.1. COLLECTION OF SAMPLES

Soil samples were collected from waste dumping grounds in Mumbai. Approximately 500 g of soil at a depth of 0-15 cm was collected in sterile jars, air dried, ground to pass through a sieve (0.2 mm), and stored at 4^0 C until further analysis.

2.2. ISOLATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM THE SOIL SAMPLE

Isolation of Phosphate solubilizing bacteria was done using readymade dehydrated Pikovskaya's media obtained from Himedia following the method described by Nautiyal (1999). Ten grams of soil was added to 100 mL of sterile saline and shaken for 2 hours to form a suspension. The suspension was diluted and 0.1 ml of appropriate dilutions were plated on Pikovskaya's agar plates containing 5g L^{-} of tricalcium Phosphate as the sole source of Phosphorus to selectively screen phosphate solubilizing bacteria. The plates were incubated at 30^0 C for three days. After 3 days of incubation, the bacterial colonies that developed a clear zone of solubilization were picked and isolated on fresh Pikovskaya's agar plates to confirm Phosphate solubilization. The resulting colonies were transferred to PVK agar slants and stored at 4^0 C.

2.3. PHOSPHATE SOLUBILIZATION IN PIKOVSKAYA'S SOLID AGAR MEDIUM

The bacterial colonies that developed a clear halo zone were investigated for Phosphate solubilization index. This was done by spot-inoculating the isolates in sterilized petri plates containing sterile Pikovsakaya's agar medium. The plates were incubated at 30^0 C for 14 days. The phosphate solubilization index was calculated by measuring the zone of solubilization and the colony diameter by using the formula Mardad et al. (2013).

$$\text{Solubilization index (SI)} = \frac{\text{Colony diameter + halo zone diameter}}{\text{Colony diameter}}$$

2.4. ESTIMATION OF PH AND PHOSPHATE SOLUBILIZATION IN PIKOVSKAYA'S BROTH

The isolates that showed a clear zone of phosphate solubilization were further selected to determine the amount of P solubilized in Pikovskaya's broth by the method described by Mihalache et al. (2018). One ml overnight grown culture was inoculated in 100 ml of Pikovskaya's broth containing Tricalcium Phosphate (TCP) as the sole source of Phosphorus and incubated at 30^0 C for 10 days. Samples were analyzed for the release every 2 days. Due to the presence of suspended insoluble tricalcium phosphate particles in the culture supernatant, it was allowed to sediment at room temperature for 15 min and then centrifuged at a very low speed (350 rpm) for 2 minutes. The residual phosphate in the culture supernatant was dissolved using 1N HCL. The amount of P solubilized was determined by Phosphomolybdate assay Murphy and Riley (1962). Pikovskaya's broth without bacterial inoculation was used as control and each treatment was prepared in triplicates. The concentration of solubilized P was determined by extrapolating with the standard graph. The initial and final pH of the broth was also determined using the digital pH meter.

2.5. IDENTIFICATION OF THE ISOLATED BACTERIA USING MATRIX-ASSISTED LASER DESORPTION/IONIZATION-TIME OF FLIGHT MASS SPECTROMETRY (MALDI-TOF-MS)

The isolated bacterial strain was identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) by the method described by Othman et al. (2019). Log score and a colour code (green, yellow, and red) were recorded for the MALDI TOF MS profiles of the isolated bacterial strain and a standard reference strain. MALDI TOF MS identification of the bacteria was done at Himedia laboratories, Mumbai.

2.6. MINERALIZATION OF ORGANIC PHOSPHATE BY THE BACTERIAL STRAIN

The bacterial strain capable of solubilizing inorganic phosphate was assessed for its organic phosphate mineralization ability following the method described by Jorquera et al. (2008). The source of organic phosphate used in Pikovskaya's medium was sodium phytate. One ml overnight grown culture was inoculated in 100 ml of Pikovskaya's broth containing sodium phytate as the sole source of Phosphorus and incubated at 37^0 C for 7 days. The amount of phosphate released in the medium was estimated by phosphomolybdate assay.

2.7. EFFECT OF DIFFERENT SALT CONCENTRATIONS ON INORGANIC PHOSPHATE SOLUBILIZATION

Pikovskaya's agar was used to assess the effect of different salt (NaCl) concentrations on the phosphate solubilizing potential of the isolated bacterial strain by the method described by Rfaki et al. (2015). Salt concentrations tested for the isolated bacterial strain were 2.5%, 5%,8%,10% and 20%. Pikovskaya's agar plates were spot inoculated with 10 µl inoculum of the isolated bacterial strain and incubated at 37^0 C for 48 hours. Phosphate solubilization was qualitatively assessed by visualizing the zone of solubilization at different salt concentrations.

2.8. EFFECT OF HEAVY METALS ON PHOSPHATE SOLUBILIZATION

The effect of three metal salts was studied on phosphate solubilization by the bacterial strain as described by Singh et al. (2015). Aluminum chloride, zinc sulphate, and lead nitrate were selected for this study. The concentration of metal salts used in this experiment was 0-1000 ppm. Stock solutions of the metal salts were prepared in distilled water and sterilized using a 0.22 um filter under aseptic conditions. Pikovskaya's agar was modified using different concentrations of the metal salts. plates were spot inoculated with 10 µl inoculum of the isolated bacterial strain and incubated at 37^0 C for 48 hours. Phosphate solubilization was qualitatively assessed by visualizing the zone of solubilization at different concentrations of heavy metals.

3. RESULTS

3.1. ISOLATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM A SOIL SAMPLE

Phosphate solubilizing bacteria was isolated from the soil sample based on the screening strategy that allowed the formation of a halo zone on the Pikovskaya's agar plates containing tricalcium phosphate as a sole source of Phosphorus. This preliminary investigation allowed the selection of an isolate exhibiting a halo zone as a phosphate-solubilizing bacteria. This was followed by growing the isolated bacteria in Pikovskaya's liquid media for 7 days which allowed estimation of solubilized phosphate with a decrease in pH of the media.

Table 1

Table 1 Isolation of Phosphate Solubilizing Bacteria

Phosphate solubilizing bacteria	Phosphate solubilization in broth (µg/ml)	Phosphate solubilization index (mm)	Initial pH	Final pH
Bacterial isolate	490	3.0	7.0	3.42
Control *S. aureus*	131	1.45	7.0	4.34

Figure 1

Figure 1 Isolated Phosphate Solubilizing Bacteria

Figure 2

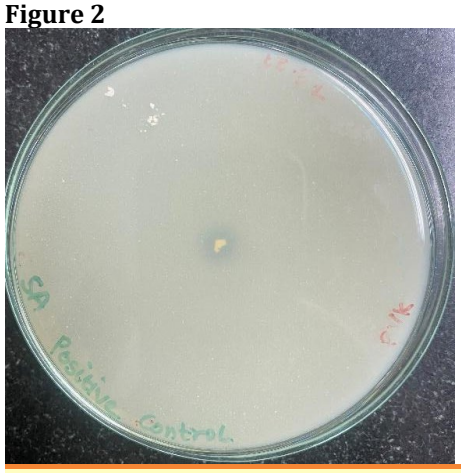

Figure 2 Positive Control *S. aureus.*

Figure 3

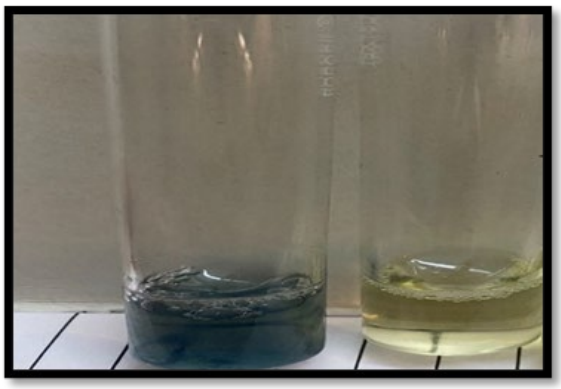

Figure 3 Blue Colour Solution in Tube 1 Indicates Positive Phosphate Solubilization
Tube 2-No Phosphate Solubilization in Uninoculated Control.

3.2. ORGANIC PHOSPHATE MINERALIZATION

Tricalcium Phosphate in Pikovskaya's broth was replaced with sodium phytate and the medium was assessed for organic phosphate mineralization by the isolated bacterial strain. The isolated bacterial strain showed the potential to mineralize phosphate from sodium phytate which was used as a source of organic phosphate.

Phosphate solubilizing bacteria	Organic Phosphate mineralization (µg/ml)
Bacterial isolate	140.22
Control *S. aureus*	66.15

3.3. EFFECT OF SALT AND HEAVY METALS ON PHOSPHATE SOLUBILIZATION

Pikovskaya's agar with different concentrations of NaCl was used to study phosphate solubilization by the isolated bacterial strain. The bacterial strain showed growth from 0-20% salt concentration. The bacterial isolate showed maximum phosphate solubilization at 2.5% salt concentration. No phosphate solubilization was seen beyond 8% salt concentration.

The isolated bacteria showed phosphate solubilization at different concentrations of metal salts ranging from 0-1000 ppm. The bacterial strain was able to solubilize phosphate even at a very high concentration of the metal salts.

3.4. IDENTIFICATION OF PHOSPHATE SOLUBILIZING BACTERIA

The isolated Phosphate solubilizing bacteria was identified at Himedia Laboratories, Mumbai using MALDI-TOF-MS. This advanced method enabled the identification of the bacterial isolate and was considered a fast and accurate method of bacterial identification. The bacterial isolate showed a score value and was identified as *Serratia rubidaea.* The score value obtained is greater than 9, representing a high degree of precision.

Bacterial samples for MALDI-TOF-MS	Score
Sample *Serratia rubidaea*	9.569
Reference *Staphylicoccus aureus* ATCC 6538	9.707

Figure 4

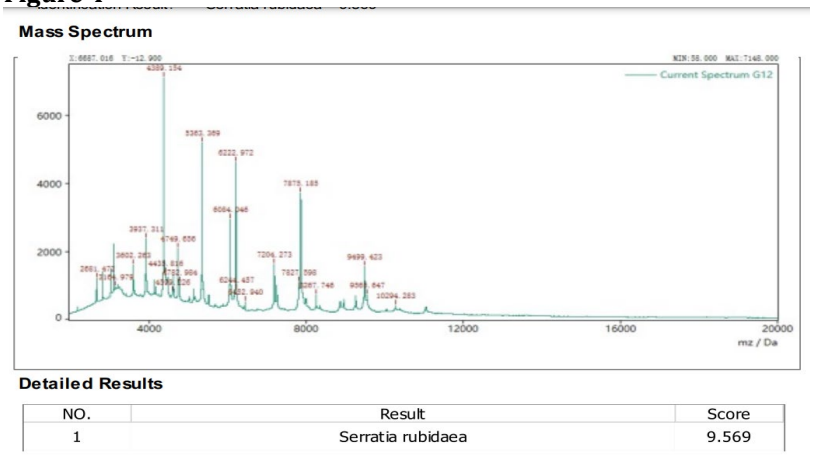

Figure 4 MALDI-TOF Sequencing

4. DISCUSSION

Phosphorus is a limiting nutrient essential for plant growth. In the past few decades, the use of biofertilizers over chemical fertilizers has gained a lot of importance. In this study, preliminary screening of the phosphate-solubilizing bacterial strain was done using Pikovskaya's media containing tricalcium phosphate as the sole phosphorus source. An efficient phosphate-solubilizing bacterial strain was selected based on its zone of solubilization. This finding is by the previously reported studies on phosphate solubilizing bacteria Hamdali (2011), Harpude et al. (2016), Sharma et al. (2011). The isolated phosphate solubilizing bacterial strain was screened by measuring the zone of phosphate solubilization and calculating the solubilization index. The zone of phosphate solubilization was found to be much higher as compared to the zone obtained for positive control *S. aureus*. The zone of solubilization obtained was greater than 5mm on Pikovskaya's agar plates. Similar findings were reported in previous studies on the isolation of phosphate-solubilizing bacteria Haouas et al. (2021). The plate assay was done to select the phosphate-solubilizing bacteria from a mixed culture plate. Quantitative estimation of the solubilized phosphate was assessed by the ability of the isolated bacterium to solubilize phosphate from tricalcium phosphate (TCP) in Pikovskaya's broth media on the 2nd, 4th, 6th,8^{th,} and 10th day. Phosphate solubilization increased till the 8th day and was found to remain constant thereafter. The maximum solubilization observed was 490 µg/ml as compared to the positive control which was 131 µg/ml. The final pH of the medium was found to decrease from 7 to 3.42. The result obtained was consistent with previous studies showing a decrease in the pH of the media by phosphate-solubilizing bacteria Alikhani et al. (2006), Zheng et al. (2018). It was observed that phosphate solubilization was associated with acidification of the media. A major mechanism associated with a decrease in pH is the secretion of organic acids by phosphate-solubilizing bacteria that chelate cations bound to phosphate compounds thereby releasing phosphate in the media Bolan et al. (1994), Mardad et al. (2013), Otani et al. (1996), Wei et al. (2018). Low molecular weight organic acids such as gluconic acid, succinic acid, acetic acid, formic acid, etc. are produced due to the oxidation of glucose by phosphate solubilizing bacteria that involves *pqq* genes Ben Farhat et al. (2009).

In most soils, a large amount of phosphate is mostly in the organic form Lidbury et al. (2021). Plants cannot utilize the organic phosphate directly, and hence it needs

to be converted to lower phosphate esters which can be available for plant uptake. Charana Walpola (2012). A major organic source of phosphorus includes phytate compounds which are abundant in the soil. Plants cannot uptake phosphate from phytate directly and hence, mineralization of phytate compounds to release phosphate is essential. This occurs by the action of enzymes like phytases and phosphonates Rawat et al. (2021). In this study, organic phosphate mineralization by the bacterium was assessed by using sodium phytate instead of tricalcium phosphate as a source of phosphate in Pikovskaya's media. The amount of phosphate mineralized by the bacteria was 140.22 µg/ml which was much higher as compared to positive control which was 66.15 µg/ml. Similar studies on the phytate mineralization potential of phosphate solubilizing bacteria were done by Jorquera et al. (2008), Qurban Ali Panhwar (2012) and Singh et al. (2014).

Environmental factors such as the presence of excess salinity and heavy metals affect the growth and phosphate solubilization ability of bacteria. Stress-tolerant bacteria are likely to be found in soil affected by different environmental stresses Nautiyal (1999). The bacterial strain identified as *Serratia Rubidaea* was isolated from waste dumping ground soil in view to study the phosphate solubilizing ability under stress conditions. In this study, the bacterial strain was exposed to varied concentrations of NaCl ranging from 0-20%. The bacterial strain showed growth on Pikovskaya's agar plate at all concentrations tested, however, the phosphate solubilization zone was observed up to 8% concentration. Similar studies were done by Son et al. (2006) and Sanjay et al. (2014). The bacterial strain showed better potential to solubilize phosphate as compared to other known phosphate solubilizing bacteria reported by Son et al. (2006) which showed phosphate solubilizing potential at not more than 3% NaCl.

In a similar study, Nakbanpote et al. (2014) demonstrated phosphate solubilization at 8% (w/v) salt concentration. Some bacterial isolates reported by Nautiyal et al. (2000) have also shown phosphate solubilization at 8% and 10% NaCl concentrations.

Phosphate solubilization and metal tolerance ability of plants help in promoting plant growth, particularly in metal-contaminated soils. Phosphate solubilization by the isolated bacteria was assessed in the presence of heavy metal salts such as aluminium chloride, lead nitrate, and zinc sulphate. The concentration of heavy metal salts used was in the range of 0-1000 ppm. The isolated phosphate solubilizing bacterium was able to solubilize phosphate at all tested concentrations.

This bacterial strain was identified as *Serratia rubidaea* by MALDI-TOF-MS with a score value of 9.56. The score value above 9.5 indicated credible and accurate identification.

5. CONCLUSION

Phosphorus is an essential element that is involved in a plethora of functional developments during plant growth. A potent phosphate-solubilizing bacterial strain was obtained from a waste dumping ground in Mumbai. The isolate was identified as *Serratia Rubidaea* by MALDI-TOF sequencing. This study demonstrated the phosphate solubilization potential of the isolated bacterial strain under stress conditions. The isolated strain *Serratia Rubidaea* showed phosphate solubilizing potential even under stress conditions making it a great candidate to be developed as a biofertilizer for use in soils under harsh environmental.

CONFLICT OF INTERESTS

None.

ACKNOWLEDGMENTS

The authors are likely to thank the Department of Life Science at Ramnarain Ruia Autonomous College for providing us with the laboratory Equipment and chemicals.

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