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PRODUCTION OF ORGANIC ACID AND SOLUBILIZATION OF INORGANIC PHOSPHATE BY A BACTERIUM ISOLATED FROM CONTAMINATED SOIL

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ABSTRACT

Many agricultural soils have significant phosphorus (P) reserves, much of which builds up because of frequent P fertilizer applications. However, roughly 95 to 99% of soil phosphorus is found as insoluble phosphates and is therefore unavailable for plant uptake. The current investigation characterized a bacterial strain that was obtained from contaminated soil and showed the ability to solubilize insoluble inorganic phosphates. An efficient phosphate-solubilizing bacterium was isolated in polluted soil in Mumbai. The phosphate solubilization index of this isolate was assessed using tribasic calcium phosphate-supplemented Pikovskaya's (PVK) medium. After growing under constant agitation for seven days, the medium pH decreased from 7.0 to 3.5 units. Based on the colony morphology, microscopic analysis, and MALDI-TOF sequencing, the bacterial isolate was identified as *Klebsiella pneumoniae*. Phosphate solubilization was linked to a pH drop caused by bacterial growth in a medium with glucose as a carbon source. The secretion of organic acids by these phosphate-solubilizing bacteria is responsible for their ability to solubilize inorganic phosphate. GC-MS analysis revealed the presence of carbamic acid, dodecanoic acid, tetra decanoic acid, and trifluoroacetic acid in the culture supernatant. The amount of phosphate solubilized by the bacterium was determined by phosphomolybdate assay and was found to be 667.0 ug/ml which was much higher than the control bacterium *S. aureus* which was 131.0 ug/ml. To the best of our knowledge, this is the first report mentioning the isolation of phosphate solubilizing bacterium from polluted soil in Mumbai.

Keywords: Phosphate Solubilization, Organic Acid Production, Tricalcium Phosphate, Phosphate Solubilization Index

1. INTRODUCTION

Phosphorus (P), is an important element that is defined as a macronutrient given that plants need relatively significant amounts of it. Additionally, it strengthens the straw of the cereal plant and the quality of the harvest, promotes flower formation and fruit production, and encourages root development, which is necessary for seed formation, maturity, and the development of disease resistance. Noorjahan et al. (2019). Although it is in its fully oxidized state as phosphate, it constantly produces many insoluble chemical complexes with calcium, iron, and

aluminum, which results in the generation of poorly soluble and inaccessible insoluble phosphate salts Mardad et al. (2013). Thus, during the past century, enormous volumes of phosphate fertilizers have been used to maximize plant yields. Large levels of P, however, quickly become immobilized and insoluble, making a significant portion of the phosphate fertilizer unavailable to plants Wang et al. (2017).

Soil microorganisms help to preserve the ecological balance by actively participating in the natural cycles of carbon, nitrogen, sulphur, and phosphorus Karpagam & Nagalakshmi (2014). Phosphate Solubilizing Microorganisms (PSMs) are a broad category of soil bacteria that are known to solubilize inorganic phosphate Mahadevamurthy et al. (2016). Numerous bacterial genera have been reported to solubilize phosphorus, including *Pseudomonas, Bacillus, Enterobacter, Azotobacter, Agrobacterium, Achromobacter, Rhizobium, Burkholderia, Flavobacterium,* and *Micrococcus*, which have been isolated from various soil types Kumar et al. (2010). Phosphate solubilizing bacteria growth is influenced by various soil characteristics, including organic matter concentration, phosphorus content, and physical and chemical characteristics Kadiri et al. (2013). It is still unclear exactly which genes underlie the bacterial phenotypic mineral phosphate solubilization in minerals. However, organic acids generated by glucose dehydrogenase are thought to be the main mechanism behind phosphate solubilization Chauhan et al. (2017). More research must be done on this topic as the synthesis of organic acids by phosphate solubilizing bacteria has not been thoroughly examined.

Phosphate-solubilizing bacteria can have positive agronomic effects, however, their abundance in the soil is not always sufficient to compete with other native bacteria. To build sustainable cropping systems, bacteria must be identified, and characterized, and crop production and economic-environmental sustainability must be prioritized Janati et al. (2022). Moreover, Waste disposal sites represent an unexplored biological niche that may support a population of microorganisms with the ability to solubilize insoluble phosphate. To better understand the solubilization mechanism, this study aimed to isolate, identify, and characterize phosphate-solubilizing bacteria (PSB) from this distinctive biotope. This was achieved by analysing the organic acids that PSB produced.

2. MATERIALS AND METHODS

2.1. COLLECTION OF SAMPLES

A sample of soil was collected from Mumbai's waste disposal sites. 500 g of soil that had been collected at a depth of 0 to 15 cm was placed in sterile jars, let to air dry, crushed to pass through a 0.2 mm screen, and kept at 40 C until additional analysis was performed.

2.2. ISOLATION OF BACTERIUM SOLUBILIZING TRICALCIUM PHOSPHATE

The phosphate-solubilizing bacterium was isolated according to the method described by Son et al. (2006). The soil sample from the waste dumping ground was suspended in a sterile saline solution and shaken for six hours. The soil samples were serially diluted and then placed on standard Pikovskaya's agar medium (glucose 10 g, MgCl2. 6 H2O 5 g, MgSO4. 7 H2O 0.25 g, KCl 0.2 g, and (NH4)2SO4 0.1 g, dissolved in 1 L distilled water). The sole phosphorus source added to the agar

medium was 5 g of tribasic calcium phosphate (TCP), which was used to selectively screen for bacteria that can release soluble inorganic phosphate from TCP. Uninoculated plates were used as controls, and the pH was adjusted to 7.0 ± 0.2. Phosphate solubilizing bacteria formed distinct zones surrounding colonies during three days of incubation at 30°C. Clear zone-encircled colonies were selected and streaked onto NBRIP plates. An efficient phosphate-solubilizing bacterium was selected based on its capacity to form a zone of clearance on PVK agar plates.

2.3. QUALITATIVE ANALYSIS OF PHOSPHATE SOLUBILIZATION ON PVK AGAR PLATES CONTAINING BROMOCRESOL PURPLE INDICATOR

Phosphate solubilization of the isolated bacterial strain was assessed by a qualitative method using a modified PVK agar medium containing bromocresol purple indicator (0.1g/l) by a method described by Vazquez et al. (2000). The bacterial isolate was spot-inoculated on a PVK bromocresol agar plate and incubated for three days at 30°C. A Color change from purple to yellow indicated positive phosphate solubilization due to a decrease in the pH of the media. By measuring the colony diameter and the zone of solubilization, the phosphate solubilization index was calculated using the formula Pande et al. (2017):

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

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

The quantitative assay for phosphate solubilization by the bacteria was performed by the method described by Rfaki et al. (2015). The bacterium showing a positive result on PVK agar media was assessed for its potential to solubilize inorganic phosphate source (Tricalcium phosphate) in PVK liquid media. 100 ml of PVK broth medium supplemented with (Ca3(PO4)2) and 100 µL of bacteria were inoculated into Erlenmeyer flasks (250 ml) for the quantitative bioassay. Autoclaved uninoculated PVK medium served as control. The flasks were incubated at 30°C on a rotating shaker with 180 rpm. Following seven days of incubation, the growth medium was centrifuged once a day for 20 minutes at 10,000 rpm. After the supernatant was decanted, the pH was measured using a pH meter, and the amount of soluble P released into the solution was measured by the ascorbic acid method. A pH meter was used in each instance to measure the pH of the supernatant.

2.5. IDENTIFICATION OF PHOSPHATE SOLUBILIZING BACTERIA

The isolated phosphate-solubilizing bacterium was identified using MALDI-TOF-MS at Himedia Laboratories in Mumbai. For both the isolated bacterial strain and a standard reference strain's MALDI TOF MS profiles, a log score, and a colour code (green, yellow, and red) were noted.

2.6. ANALYSIS OF ORGANIC ACIDS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

The gas chromatography-mass spectrometry method by Kaur et al. (2018) was used for the analysis of organic acids produced by the phosphate solubilizing bacterium in the broth method. The supernatant of the phosphate solubilizing bacterium grown in PVK medium was taken after centrifuging at 13000 rpm for 15 min. One ml of the oven-dried samples was used to extract the organic acids, and 0.5 ml of 0.5 N HCl and 0.5 ml of methanol were added. Following this, the samples were centrifuged at 12,000 rpm for 10 minutes after being shaken for up to three hours. The mixture was incubated for 6 hours at 60°C in a water bath after the supernatant, 300 µL of methanol, and 100 µL of 50% sulfuric acid were added. The supernatant was cooled to 25 °C before 800 µL of chloroform and 400 µL of distilled water were added, and the mixture was vortexed for one minute. The amount of organic acid in the chloroform was determined by analysing its lower layer. The GC-MS of the samples was done at Dr. P.S. Ramanathan Advanced Instrumentation Centre, Ramnarain Ruia College, Matunga, Mumbai-400019. The organic acids were detected and identified by comparing the peak areas and retention times of their chromatograms with their references in the database.

3. RESULTS

3.1. ISOLATION OF BACTERIUM ABLE TO USE TRICALCIUM PHOSPHATE AS A SOLE SOURCE OF PHOSPHORUS

Soil samples from dumping grounds were collected from a municipal dumping ground in Mumbai to analyze samples that were most affected by the dumping of industrial, agricultural, and domestic wastes. Organisms that can solubilize phosphate produce a distinct zone surrounding their colonies, indicating that the microorganisms are phosphate-solubilizing bacteria. An efficient phosphate-solubilizing bacterium was selected based on its ability to solubilize phosphate and form a zone of solubilization of PVK agar media.

Figure 1

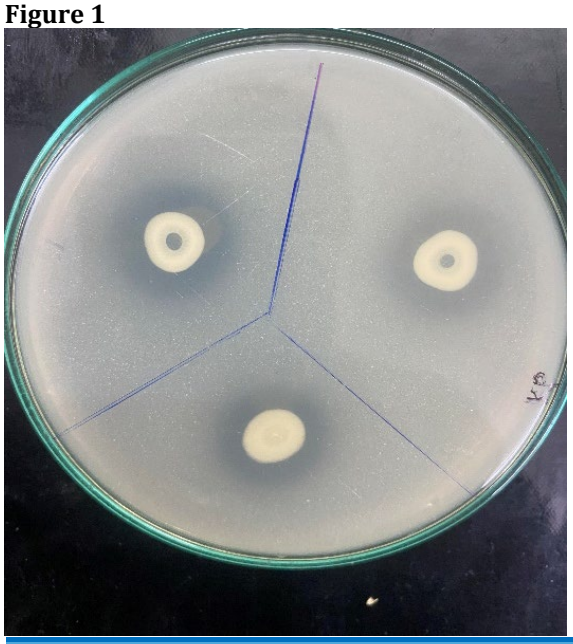

Figure 1 Isolated Phosphate Solubilizing Bacteria

Figure 2

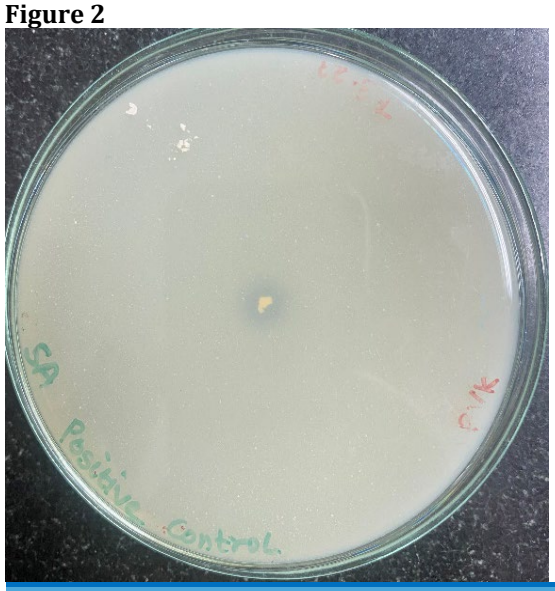

Figure 2 Positive Control S. Aureus.

The phosphate solubilization index (PSI) of the isolated bacterium and positive control bacterium *S. aureus* was found to be 3.33 and 1.45 respectively

3.2. QUANTITATIVE ESTIMATION OF PHOSPHATE SOLUBILIZATION IN PIKOVSKAYA'S BROTH MEDIUM

The isolated bacterial strain's capacity to solubilize phosphate was tested in broth culture. The results demonstrated that the amount of soluble P in the supernatants of the bacterial strain was 667.0 µg/ml after five days of incubation. The amount of phosphate solubilized by the positive control strain S. aureus was found to 131.0 µg/ml be. It was observed that the amount of soluble phosphate increased as the final pH of the broth media decreased from 7.0 to 4.30.

Figure 3

Figure 3 Blue Colour Solution Indicates Positive Phosphate Solubilization an Uninoculated PVK Medium Was Used as a Control.

3.3. QUALITATIVE ANALYSIS OF PHOSPHATE SOLUBILIZATION USING BROMOCRESOL PURPLE INDICATOR DYE

The isolated phosphate solubilizing bacterial strain was tested for an additional phosphate solubilization test using a bromocresol indicator in pikovskaya's agar medium. A yellow zone of solubilization around the inoculated bacterium indicated positive phosphate solubilization.

Figure 4

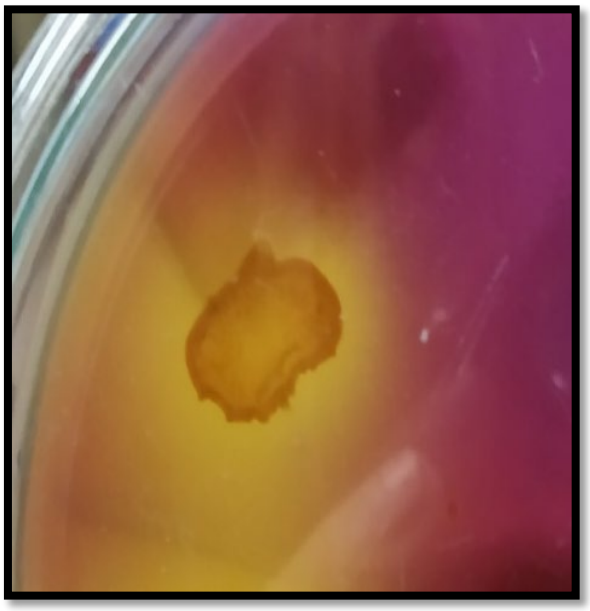

Figure 4 The Yellow Zone Around the Colony Represents Phosphate Solubilization.

3.4. IDENTIFICATION OF PHOSPHATE SOLUBILIZING BACTERIA

Using MALDI-TOF-MS, the isolated phosphate solubilizing bacterium was identified at Himedia Laboratories in Mumbai. This sophisticated technique made it possible to identify the bacterial isolate and was regarded as a quick and precise way to identify bacteria. *Klebsiella pneumoniae* was identified as the bacterial isolate that displayed a score value of 9.662. With a score value higher than 9, a high level of precision is indicated.

Table 1

Table 1 MALDI-TOF Sequencing Score Values

Bacterial samples for MALDI-TOF-MS	Score
Klebsiella pneumoniae	9.662
Reference *Staphylicoccus aureus* ATCC 6538	9.707

Figure 5

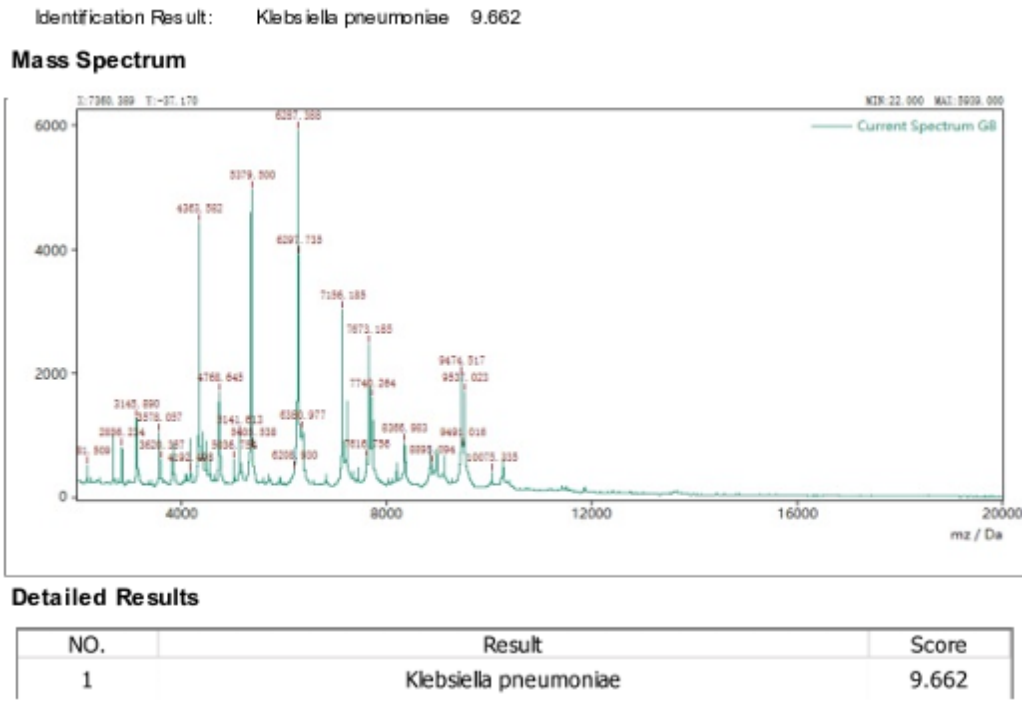

Figure 5 MALDI-TOF Sequencing

3.5. DETECTION OF ORGANIC ACIDS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

Organic acid production by the isolated phosphate solubilizing bacterium was investigated in PVK broth culture. GS-MS analysis of the culture filtrates revealed the presence of several organic acids that confer the capacity to solubilize insoluble TCP to the phosphate-solubilizing bacterial strain. The organic acids in the culture supernatant were found to be carbamic acid, dodecanoic acid, tetra decanoic acid, and trifluoroacetic acid.

Figure 6

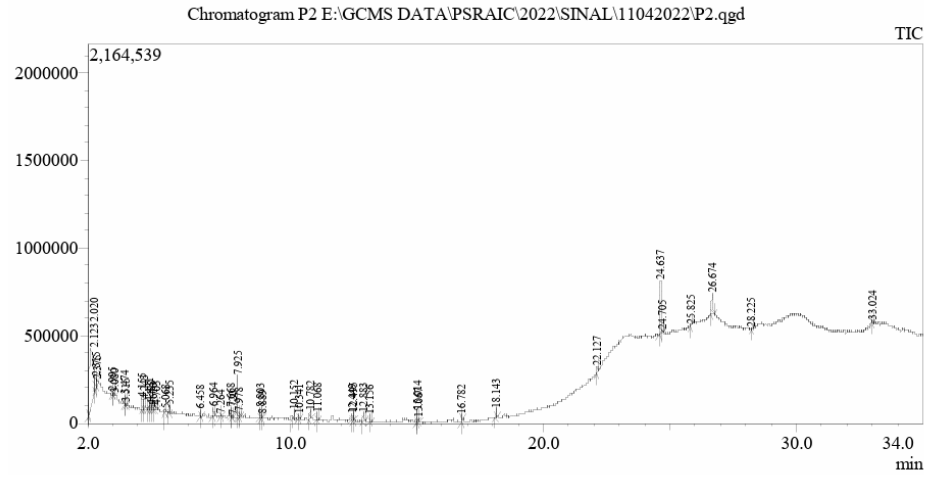

Figure 6 GC-MS Chromatogram of Organic Acid Analysis

Figure 7

1	2.020	401124	4.50	533934	(S)-(+)-1-Cyclohexylethylamine
2	2.123	3635038	40.81	334585	Carbamic acid, monoammonium salt
3	2.315	215622	2.42	70377	Methanol, (4-amino-1,2,5-oxadiazol-3-yl)(imino)-
4	2.375	81533	0.92	20088	Benzeneethanamine, 2,5-difluoro-.beta.,3,4-trihydro
5	2.995	19675	0.22	17254	Cyclopropane, 1-heptyl-2-methyl-
6	3.080	32087	0.36	12107	Acetamide, 2,2-dichloro-
7	3.474	57988	0.65	55939	Decane, 3,7-dimethyl-
8	3.515	16972	0.19	14920	Octane, 5-ethyl-2-methyl-
9	4.155	211245	2.37	69021	6-Octen-1-ol, 3,7-dimethyl-, (R)-
10	4.243	293026	3.29	58425	6-Octen-1-ol, 3,7-dimethyl-, (R)-
11	4.421	93612	1.05	42110	1-Dodecanol
12	4.488	80653	0.91	26370	Dodecane
13	4.610	42212	0.47	12758	1-Decanol, 2-methyl-
14	4.703	62730	0.70	24555	6-Octen-1-ol, 3,7-dimethyl-, formate
15	5.068	35990	0.40	21761	Decane, 3,7-dimethyl-
16	5.235	60792	0.68	46395	Nonane, 5-butyl-
17	6.458	66907	0.75	41238	3-Hexadecene, (Z)-
18	6.964	134957	1.52	54003	(-)-Aristolene
19	7.264	13948	0.16	13044	Hexadecane
20	7.668	92217	1.04	49496	Heptadecane, 8-methyl-
21	7.720	18416	0.21	12911	Behenyl chloride
22	7.925	374825	4.21	237576	Phenol, 2,4-bis(1,1-dimethylethyl)-
23	7.978	98783	1.11	26505	Dodecanoic acid, methyl ester
24	8.803	98517	1.11	58264	1-Pentadecene
25	8.889	37791	0.42	19885	Hexadecane
26	10.152	77128	0.87	48796	Eicosane
27	10.341	51691	0.58	27364	Methyl tetradecanoate
28	10.782	128153	1.44	50304	Tetradecanoic acid

Figure 7 GC-MS Report of Organic Acid Analysis

Figure 8

Peak#	R.Time	Area	Area%	Height	Name
29	11.068	75232	0.84	42170	1-Nonadecene
30	12.449	76753	0.86	36743	Eicosane
31	12.495	93759	1.05	47465	Hexadecanoic acid, methyl ester
32	12.883	135615	1.52	50482	n-Hexadecanoic acid
33	13.156	66750	0.75	40976	1-Nonadecene
34	15.014	122400	1.37	66916	Hexadecanoic acid, butyl ester
35	15.067	45058	0.51	23910	Heneicosyl trifluoroacetate
36	16.782	67745	0.76	35784	Octadecanoic acid, butyl ester
37	18.143	91458	1.03	47802	Diisooctyl phthalate
38	22.127	75431	0.85	34769	.alpha.-Amyrin
39	24.637	958439	10.76	306815	Silane, diethylheptyloxyoctadecyloxy-
40	24.705	2099	0.02	5125	1,2-Benzisothiazol-3-amine tbdms
41	25.825	20072	0.23	10084	1,2-Bis(trimethylsilyl)benzene
42	26.674	505466	5.67	122597	Pentacyclo[19.3.1.1(3,7).1(9,13).1(15,19)]octacosa-
43	28.225	15310	0.17	11191	tert-Butyl(5-isopropyl-2-methylphenoxy)dimethylsil
44	33.024	21838	0.25	13112	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-
		8907057	100.00	2895926	

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Figure 8 GC-MS Report of Organic Acid Analysis

4. DISCUSSION

Plant nutrient models have long demonstrated that slow diffusion of inorganic phosphate (Pi) is a major barrier to P acquisition by plants. Soil P is characterized by its restricted mobility when compared to other important nutrients, such as nitrogen or potassium Janati et al. (2022). In the present study, Pikovskaya's media, which utilizes tricalcium phosphate as the only source of phosphorus, was used for the initial screening of the phosphate-solubilizing bacterial strain. Selecting an effective phosphate-solubilizing bacterium is essential because it practically raises P in the rhizosphere of plants. Based on its zone of solubilization, an effective phosphate-solubilizing bacterial strain was isolated from a waste-dumping ground

soil sample in Mumbai. Phosphate solubilization based on its zone of solubilization in pikovskaya's medium has been previously reported by De Campos et al. (2016). The zone of solubilization for the isolated phosphate solubilizing bacterium and a positive control bacterium *S. aureus* was measured and was found to be 3.33 and 1.45 respectively. When compared to the zone obtained for the positive control *S. aureus*, it was found that the zone of phosphate solubilization was significantly higher. 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).

Another qualitative assay to confirm phosphate solubilization was done using bromocresol purple indicator dye in pikovskaya's agar medium. The plate was observed to produce a yellow zone around the bacterial colony Umeh & Sapele (2015). The appearance of a yellow zone of coloration around the bacterial colony is indicative of a decrease in pH due to the production of acids by the bacterium. Similar findings have been reported by Zheng et al. (2018).

Quantitative estimation of phosphate solubilization was carried out using the isolated phosphate solubilizing bacterial strain on a PVK liquid medium for 10 days. The phosphate solubilizing ability of the strain was increased from 0.0 mg/ml to 667.0 mg/ml as the pH decreased from 7.0 to 4.30. Similar findings were reported by Cao et al. (2018), Henri et al. (2008). Acidification of the media was shown to relate to phosphate solubilization. A primary factor linked to a drop in pH is the release of organic acids by bacteria that solubilize phosphate, which releases phosphate into the media by chelating cations attached to phosphate molecules Bolan et al. (1994), Mardad et al. (2013), Otani et al. (1996), Wei et al. (2018). Low molecular weight organic acids involving pqq genes are produced when phosphate solubilizing bacteria oxidize glucose. Some of these acids typically include gluconic acid, succinic acid, acetic acid, and formic acid. Ben Farhat et al. (2009). The isolated phosphate solubilizing bacterial strain demonstrated the production of organic acids such as carbamic acid, dodecanoic acid, tetra decanoic acid, and trifluoroacetic acid by GC-MS analytical technique. The findings of this study are like a previously reported study on organic acid production such as oxaloacetic acid, succinic acid, acetic acid, isovaleric acid, and caproic acid by phosphate solubilizing bacteria reported by Vazquez et al. (2000). The simultaneous production of different organic acids by the phosphate-solubilizing bacteria isolated in this study may enhance their potential for solubilizing insoluble phosphate.

This bacterial strain was identified as *Klebsiella pneumoniae* by MALDI-TOF-MS with a score value of 9.66. The score value above 9.5 indicated credible and accurate identification.

5. CONCLUSION

The main objective of this study was to isolate and identify phosphate solubilizing bacterium from the waste dumping ground in Mumbai. Phosphorus molecules become unavailable for plant uptake due to inorganic P fixation from insoluble complexes. Utilizing biofertilizers, like microbes, can aid with this by sustainably promoting plant development. Based on the above results, an efficient phosphate-solubilizing bacterium *Klebsiella pneumoniae* was isolated and evaluated for its phosphate-solubilizing potential. The organic acid analysis using GC-MS confirmed the presence of organic acids responsible for phosphate solubilization. To fully utilize these bacteria, further research on microbial-mineral interactions and genetic pathways is required. New innovative studies should concentrate on the ways that microbial biotechnology may be applied in agriculture to find new

phosphate-solubilizing bacteria that can be used in a consortium to have competent microbial inoculants in systems for producing sustainable cultures under different circumstances.

CONFLICT OF INTERESTS

None.

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