

Organic Acid Profiles of Phosphate Solubilizing Bacterial Strains in the Presence of Different Insoluble Phosphatic Sources Under *In vitro* Buffered Conditions

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Abstract

The production of weak organic acids by microorganisms has been attributed as the prime reason for the solubilization of insoluble phosphates under both *in vitro* and soil conditions. Literature seems to be heavily biased towards gluconic acid production by microbes and its subsequent release into the environment as the key factor responsible for phosphate solubilization. This has found credibility since gluconic acid being a product of the Krebs's cycle is often detected in large quantities in the culture media, when assayed under *in vitro* conditions. In the present work, the organic acid profiles of four elite phosphate solubilising isolates were determined in the presence of different insoluble sources of phosphates, under *in vitro* buffered culture conditions by HPLC (High-Performance Liquid Chromatography). While most previous studies did not use a buffered culture media for elucidating the organic acid profile of phosphate solubilizing bacterial isolates, we used a buffered media for estimation of the organic acid profiles. The results revealed that apart from gluconic acid, malic acid is produced in significant levels by phosphate solubilizing bacterial isolates, and there seems to be a differential pattern of production of these two organic acids by the isolates in the presence of different insoluble phosphate sources.

Keywords: Phosphate Solubilising Bacteria, Organic Acids, Malic acid, Gluconic acid

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INTRODUCTION

Phosphorus is an essential nutrient for plant growth and reproduction. Phosphorus is available in soil primarily in organic or inorganic forms, but most forms are not available for plant uptake due to its tendency to form complexes with cations such as Calcium (Ca) in alkaline soils and Aluminum (Al) and Iron(Fe) in acidic soils. Several studies have advocated the use of biological formulations to overcome P deficiency in soil. Many rhizospheric microorganisms have been reported to solubilize mineral phosphates by different mechanisms. The most commonly encountered phosphate solubilizing bacterial genera are *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter*. Their ability to solubilize phosphates has been mainly attributed to their organic acid secretion potential^{1,2}.

One of the most well-known mechanisms of phosphate solubilization is the release of weak organic acids by phosphate solubilizing microorganisms, which solubilize the elemental-phosphatic complexes thereby releasing the solubilized phosphorus into the culture media. Most often different divalent and trivalent organic anions such as malate, citrate and oxalate are produced by the microbes and are implicated to play an important role in the solubilization of phosphates³. This observation has been supported by several studies on the organic acid production profile under *in vitro* culture conditions⁴⁻⁷. Under *in vitro* culture conditions, the production of organic acids by bacterial strains is largely influenced either by the growth medium or by the presence of insoluble phosphate sources. Gluconic acid has been reported as the main organic acid secreted by many phosphate solubilizing microorganisms under laboratory conditions and is well known for its chelation abilities^{8,9}. Gluconic acid is produced by the direct extra-cellular oxidation of glucose catalysed by enzyme glucose dehydrogenase coupled with the co-factor pyrroloquinoline quinone (PQQ)^{10,11}. The oxidation of gluconic acid gives rise to the production of 2-ketogluconic acid, a substantial carboxylic acid that can chelate calcium ions and dissolve hydroxyapatites¹². Though there have been reports of other organic acids viz., oxalic acid, citric acid, acetic acid, malic acid, succinic acid, tartaric acid, propanoic acid, lactic acid, fumaric

acid, pyruvic acid etc^{9,13-18} being produced by phosphate solubilizing bacterial strains, gluconic acid has gained much importance due to its ability to solubilise phosphates under *in vitro* conditions^{12,16,18-20}.

Arvind & Gulati¹³ were the first to decipher the organic acid profile of phosphate solubilizing Pseudomonads using multiple insoluble substrates. But this study was carried out in a non-buffered medium. They presented a detailed description of organic acids produced by different strains of *Pseudomonas* that solubilised Udaipur rock phosphate (URP), Mussorie rock phosphate (MRP) and North Carolina rock phosphate (NCRP) under non-buffered *in vitro* conditions and concluded that gluconic acid and 2-ketogluconic acid are the major organic acids responsible for phosphate solubilization under *in vitro* conditions. A positive correlation between the levels of phosphate solubilisation and the quantity of gluconic acid produced by *Enterobacter cloacae* EB 27, *Serratia marcescens* EB 67, *Serratia sp.* EB 75, *Pseudomonas sp.* CDB 35 and *Pseudomonas sp.* BWB 2 was established by Hameeda et al²¹. The bacterium *Azospirillum* has been reported to release gluconic acid at high concentrations under *in vitro* conditions which was implicated in the solubilization of phosphates¹⁴.

While several studies in the past have focussed on utility of gluconic acid in phosphate solubilization, they seem to have missed the significant levels of another important organic acid viz., malic acid in the solubilization of phosphates. The present study highlights the extra cellular release of malic acid in significant quantities by strains of three different bacterial genera viz., *Pseudomonas*, *Bacillus* and *Paraburkholderia* species under *in vitro* conditions. The isolates were carefully chosen from a collection of phosphate solubilizing microbes in order to represent the three major soil genera that play a vital role in phosphate solubilization in the soil. Malic acid has been reported as a crucial metal chelator that is released majorly by fungal species such as *Aspergillus niger* while solubilizing rock phosphate under *in vitro* conditions²⁰. There are very few reports about the utility of malic acid production in significant levels by bacterial species and its utility in phosphate solubilisation, nor is its mechanism of phosphate solubilization has been

clearly defined. Also concentrations of malic acid equivalent to or at par to gluconic acid have never been reported. This study mainly focuses organic acid production profiles of elite bacterial isolates in a buffered medium under *in vitro* conditions in the presence of three different insoluble substrates *viz.*, tricalcium phosphate (TCP), Aluminium phosphate Al-P and Ferrous phosphate Fe-P.

MATERIALS AND METHODS

Bacterial strains and determination of the phosphate solubilization potential of the isolates

Four efficient phosphate solubilizing bacterial isolates were included in the present study *viz.* *Pseudomonas sp* (BAN-4) isolated from Banana (*Musa sp.*) rhizosphere, *Pseudomonas sp* (DIN-5) isolated from Sapota (*Manilkara zapota*) rhizosphere, *Bacillus sp* (NE-4) isolated from Rice (*Oryza sativa*) and *Paraburkholderia tropica* (P-31) isolated from Pomegranate (*Punica granatum*) rhizosphere. These strains were characterized based on their phenotypic characters and 16S rRNA gene sequencing. The bacterial strains were maintained at -70°C in 50 % glycerol stocks.

The phosphate solubilization potential of the elite bacterial strains was studied using three insoluble phosphate sources *viz.*, Tricalcium phosphate (TCP), Aluminium phosphate Al-P, Iron phosphate Fe-P in a buffered medium. The media contained 100 mM glucose, 25 µM Magnesium sulphate (MgSO₄), 10 mM Ammonium chloride (NH₄Cl) and the following micronutrients (mg/l) *viz.*, Ferrous sulphate (FeSO₄)-3.5; Zinc sulphate (ZnSO₄)-0.16; Copper sulphate (CuSO₄)-0.08; Boric acid (H₃BO₃)- 0.5; Calcium chloride (CaCl₂) - 0.03; Manganese sulphate (MnSO₄) - 0.4 and it was buffered with 100 mM Tris Hydrochloride (HCl) (pH 8.0)²²⁻²⁴. The elite bacterial strains were grown in 100 ml minimal buffered broth supplemented with either 0.5% tricalcium phosphate (TCP) or Aluminium phosphate (Al-P) or Ferrous phosphate (Fe-P) separately and incubated at 30°C for 7 days under shaking conditions. On completion of the incubation period the culture was centrifuged at 10,000 rpm for 10 min and the pH of the culture supernatant was recorded. Subsequently the soluble P released by the bacterial isolates was determined quantitatively by the spectrophotometric method²⁵. All the studies were conducted in triplicate.

Identification and quantification of the organic acid profile of the promising isolates under using high performance liquid chromatography (HPLC)

The cell free culture supernatant obtained mentioned above was filter sterilized using a 0.22 µm nylon filter. The detection and quantification of organic acids was done using a HPLC (Shimadzu, Japan), equipped with photodiode array detector (SPD-M20A) and Synergi 4µm RP-C18 column (Phenomenex, USA, 250X4.6mm). The mobile phase consisted of 1mM Sulphuric acid (H₂SO₄) + 8mM Sodium sulphate (Na₂SO₄) (1:1v/v) containing 0.1% orthophosphoric acid at 0.5ml/min flow rate. The organic acids present in the culture filtrate were quantified at 210 nm using standards of different organic acids *viz.*, tartaric acid, formic acid, malic acid, malonic acid, lactic acid, citric acid, succinic acid, propionic acid, gluconic acid, oxalic acid, Keto-D glutarate. The eluates were detected and quantified with reference to the peak areas obtained by using the respective standards.

RESULTS AND DISCUSSION

Phosphate solubilizing potential of the bacterial strains

Among four strains *Paraburkholderia tropica* P-31 released the highest levels of soluble P (50.0 µg/ml) when TCP was used as insoluble source of phosphorus and drop in pH was observed from 7.0 to 4.4. The isolate *Bacillus sp.* NE-4 released the highest levels of soluble P (19.0 µg/ml) when Fe-P was used as insoluble source of phosphorus and a drop in pH was observed from 7 to 5.0. *Pseudomonas sp* DIN-5, released the highest levels of soluble P i.e. 21.9 (µg/ml) when Al-P was used as insoluble source of phosphorus in the medium but a concomitant drop in pH was not observed (Table -1). It should be noted here that solubility of Calcium phosphate (Ca- P) increases exponentially with decreasing pH whereas solubility of Iron phosphate (Fe-P) decreases with the decrease in the pH in the range of 4.5 to 3.5 and solubility of Aluminum Phosphate (Al-P) is lowest in the pH range of 5.5-4.5²²⁻²⁴. The results of the present study are therefore in accordance with the observations of Merbach²³ and Henry²⁴. Microorganisms have been known to deploy different mechanisms for the dissolution of different phosphate complexes. Acidification of the medium is proposed as a major mechanism for the

dissolution of the Ca-P complex, this mechanism results in the acidification of the medium, due to the release of protons. Alternatively metal complexing and metal reduction are the possible mechanisms of dissolution of Al and Fe phosphates. By forming metal complexes the

Table 1. Quantitative estimation of the P solubilising abilities of the elite phosphate solubilising bacterial isolates under *in vitro* conditions

Isolate	TCP		Fe-P		Al-P	
	pH	Soluble P (µg/ml)	pH	Soluble P (µg/ml)	pH	Soluble P (µg/ml)
<i>Pseudomonas</i> sp.(DIN-5)	4.6	46.4	7.3	6.0	7.4	21.9
<i>Paraburkholderia tropica</i> (P-31)	4.4	50.0	7.3	6.7	7.3	8.2
<i>Bacillus</i> sp.(NE-4)	5.2	20.3	5.0	19.0	5.0	6.3
<i>Pseudomonas</i> sp. (BAN-4)	4.6	35.6	5.2	1.0	4.8	7.3

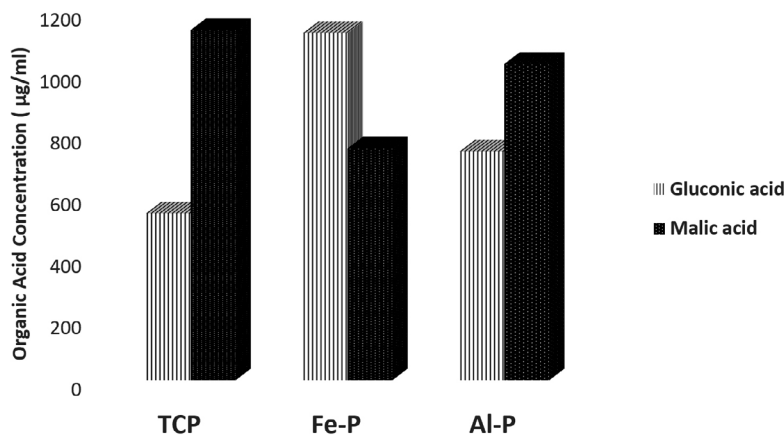


Fig. 1(a). Relative concentrations of malic and gluconic acids produced by the phosphate solubilizing *Pseudomonas* sp. DIN-5 under *in vitro* conditions in the presence of different insoluble phosphate sources

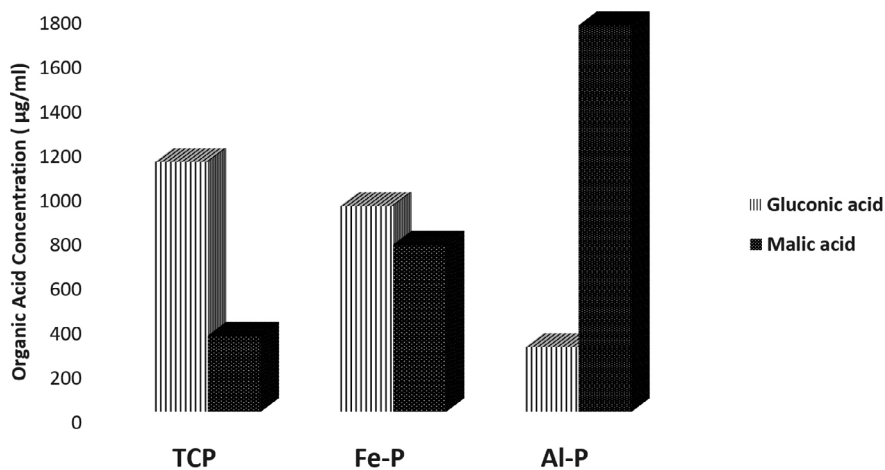


Fig. 1 (b). Relative concentrations of malic and gluconic acids produced by the phosphate solubilizing *Bacillus* sp. NE-4 under *in vitro* conditions in the presence of different insoluble phosphate sources

organic acids reduce metals from their variable oxidation states to lower oxidation states thereby resulting in the release of soluble P forms²⁶.

There are many reports of PSB's which solubilize TCP but do not dissolve Fe-P and Al-P complexes and are therefore of not much utility under dynamic field conditions^{27,28} because of two major reasons, viz., a contrasting range of soil pH levels and is the strong buffering capacity of soils²⁹. The experimental framework of the present study therefore took cognizance of the above factors. Though, buffering of media drastically lowers the phosphate solubilizing capacity of microbes the elite phosphate solubilisers are

likely to sustain their performance under buffered conditions³⁰.

Deciphering the organic acid profile of the phosphate solubilizing bacterial isolates under *in vitro* conditions by High Performance Liquid Chromatography (HPLC)

When the organic acid produced by the isolates were quantified under *in vitro* conditions, the concentration of organic acids viz., gluconic acid and malic acid were found to be higher compared to other acids (Table 2). Apart from and gluconic acids, succinic acid was the other predominant organic acid produced across substrates and isolates. Closer scrutiny of the

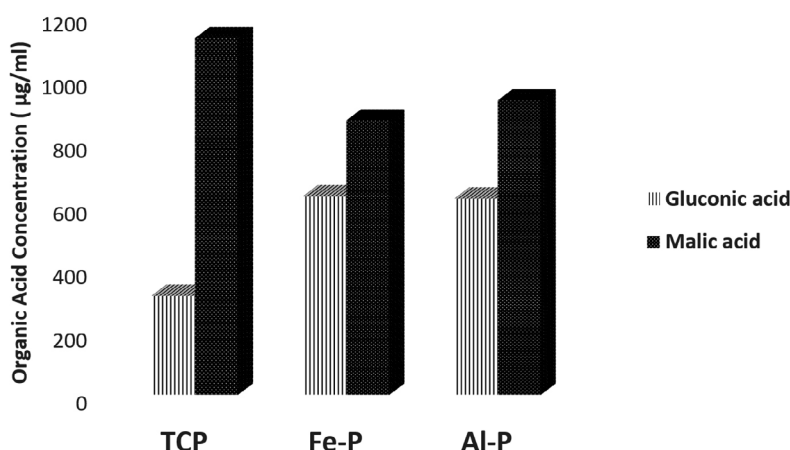


Fig. 1 (c). Relative concentrations of malic and gluconic acids produced by the phosphate solubilizing *Pseudomonas* sp. BAN-4 under *in vitro* conditions in the presence of different insoluble phosphate sources

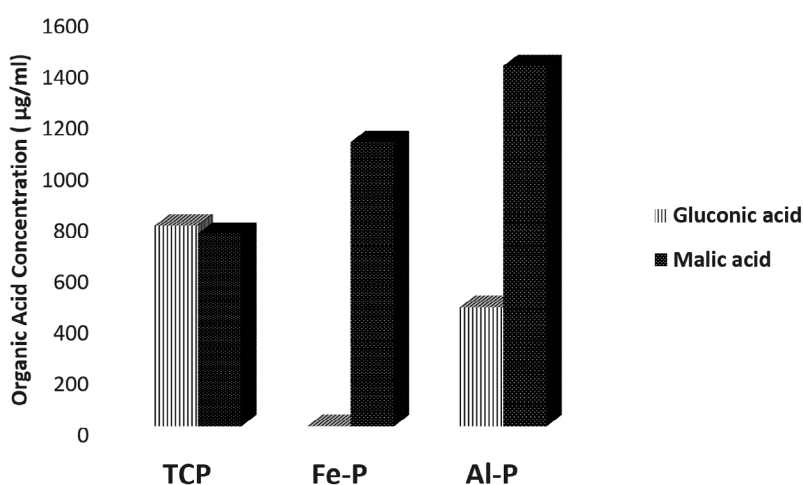


Fig. 1 (d). Relative concentrations of malic and gluconic acids produced by the phosphate solubilizing *Paraburkholderia* tropica. P-31 under *in vitro* conditions in the presence of different insoluble phosphate sources

Table 2. Organic acid profile of the elite isolates under *in vitro* conditions

Bacterial Isolates	Insoluble P sources	Organic Acid Concentration (µg/ml)										
		Tartaric acid	Formic acid	Malic acid	Malonic acid	Lactic acid	Citric acid	Succinic acid	Propionic acid	Gluconic acid	Oxalic acid	Keto D Glutarate
<i>Pseudomonas sp</i> DIN-5	TCP	20.2	19.9	1135.4	865.6	ND	83.8	213.9	ND	541.4	ND	ND
	Fe-P	18.9	16.6	751.9	91.3	92.5	91.4	652.7	ND	1125.4	ND	ND
	Al-P	ND	86.3	1025.7	ND	ND	62.9	262.8	ND	742.3	ND	36.9
<i>Bacillus sp</i> NE-4	TCP	42.8	20.5	342.5	92.2	ND	76.2	552.7	ND	1124.4	ND	52.7
	Fe-P	35.7	9.3	752.2	ND	85.3	ND	747.9	ND	926.5	ND	ND
	Al-P	23.8	8.1	1739.0	423.2	ND	89.4	572.3	ND	292.1	5.5	141.5
<i>Pseudomonas sp</i> BAN-4	TCP	38.9	41.1	1124.9	193.6	84.4	ND	553.6	ND	312.1	ND	92.6
	Fe-P	23.9	41.3	865.3	711.4	65.3	76.6	785.5	ND	625.9	2.7	23.6
	Al-P	52.6	15.4	928.8	212.5	91.5	235.6	411.2	ND	618.9	ND	21.1
<i>Paraburkholderia tropica</i> P-31	TCP	37.9	58.4	752.3	547.9	ND	52.9	411.6	ND	784.3	5.3	37.9
	Fe-P	41.6	19.8	1110.5	835.8	ND	21.7	112.7	ND	0.5	5.6	41.6
	Al-P	96.2	23.1	1408.1	ND	22.6	131.4	841.4	ND	465.9	ND	96.2

ND- Not detected

data revealed a differential pattern of organic acid release by the isolates in the presence of different insoluble sources of phosphates. The extracellular concentrations of malic acid were higher when *Pseudomonas sp.* (DIN-5) was cultured in the presence of TCP and Al-P whereas gluconic acid was detected in higher concentrations when Fe-P was used as an insoluble substrate of P. Conversely in the case of *Bacillus sp.* (NE-4) the gluconic acid concentrations were higher in the presence of TCP and Fe-P while extracellular malic acid levels were higher when Al-P was used as an insoluble substrate of P. The strain *Pseudomonas sp.* (BAN-4) released highest concentrations of malic acid compared to gluconic acid across insoluble substrates of P, while *Paraburkholderia tropica* (P-31) released highest levels of malic acid when Fe-P and Al-P were used as insoluble substrates of P, while the highest concentrations of gluconic acid was recorded when TCP was used as an insoluble source of P. It could be concluded that the highest levels of malic acid were detected in eight of the twelve scenarios under which the organic acid profiles were estimated (Fig. 1 a, b, c, d).

The earlier studies on the *in vitro* organic acid production by phosphate solubilizing bacterial isolates have reported that gluconic acid is produced by several bacterial genera in significant quantities while malic acid is produced by phosphate solubilizing fungal genera like *Aspergillus*^{1,20,31-35}. The novelty of this study rests in the fact that it has been proved conclusively that bacterial genera apart from *Pseudomonas* produce malic acid in higher or equivalent quantities to that of gluconic acid across insoluble phosphate sources in a buffered media.

In general malic acid has applications in the liquor, food, synthetic and pharmaceutical industries³³ but its agricultural usage is not well known. L-malic acid is known to be produced majorly by species of *Aspergillus*, *Schizophyllum*³⁴⁻³⁶, *Penicillium*, *Aureobasidium*^{1,30,34}, an engineered strain of *E.coli*³⁵, species of *Penicillium*, *Aureobasidium*³⁶⁻⁴⁰, and *Bacillus subtilis*^{35,41}. The literature suggests that the most probable mechanism of malic acid production is the increase in utilisation of glucose via carboxylation of pyruvate, followed by reduction of oxaloacetate³⁶. *Saccharomyces cerevisiae* isolated from Sake

mash was reported to produce very high levels of malic acid by downregulating the production of thymine genes (*THI4*) and upregulation of stress genes (HSP12)³⁷. Similarly among bacteria a mutant strain of *Thermobifida fusca muC* accumulated malic acid on a medium containing cellulose where phosphoenolpyruvate is known to get converted to oxaloacetate in the presence of phosphoenol carboxylase and further oxaloacetate being reduced to malate in the presence of malate dehydrogenase³⁹⁻⁴¹. Microorganisms utilising the reductive pathway are known to secrete maximum concentrations of malic acid qualitatively and quantitatively⁴²⁻⁴⁴. Fungal strains of *Aspergillus*, *Ustilago trichophora* and *Aureobasidium pullulans* are known to secrete good quantities of malic acid from biofuel related coproducts and biomass⁴². Alternatively, other methods have been mentioned in literature for the increased synthesis of malic acid like in case of *Saccharomyces cerevisiae* whose cells contained overexpressed gene for fumarate hydratase catalysed the conversion of fumarate to malate⁴³.

Several enzymes are known to play a role in metabolising malic acid. The NADP-malic enzyme (NADP-ME), has been known to produce the essential components of energy and for the biosynthesis of defense components. These defense compounds are implicated in the malate metabolism in plant defense⁴⁴. Malic enzymes have also been found in *Sinorhizobium meliloti*^{45,46}, *Bacillus stearothermophilus*,⁴⁷ *Streptococcus bovis*⁴⁸, *Corynebacterium glutamicum*⁴⁹, *Lactococcus lactis*⁵⁰ and *B. subtilis*^{51,52}. The list is exhaustive when it comes industrial production of malic acid and its utility but from the agriculture point of view, we have seen two main studies, one is based on metal chelation and other is known for eliciting plant defences, yet another study focusses on demonstrating the role of malic acid released from the roots of Arabidopsis (*Arabidopsis thaliana*) which attracts the useful rhizobacterium *Bacillus subtilis* FB17 and assisted in biofilm formation⁵³. Thus far, no study demonstrated the possible pathway of malic acid production by Gram negative bacteria which is good enough to bring about the solubilization of phosphates. While gluconic acid is known as one of the key organic acids released by several species of *Pseudomonas*⁵⁴ and many Gram negative

and Gram positive bacterial genera and fungal genera for phosphate solubilization⁵⁵⁻⁶⁰. The role of malic acid in phosphate solubilization has not been well documented. This study opens up the exploration of the utility and role of malic acid is the solubilization of phosphates, under *in vitro* conditions.

CONCLUSION

This study has shown that under *in vitro* buffered conditions apart from gluconic acid, malic acid is also produced in significant levels by the Gram negative phosphate solubilizing bacterial isolates belonging to various genera. There seems to be a differential pattern of the production of these two major organic acids across isolates and insoluble sources of phosphates. But nevertheless in eight of the twelve scenarios explored in this study, malic acid concentrations were higher than the gluconic acid concentrations, thereby conclusively establishing the role of malic acid in phosphate solubilization. Previous studies have not determined the possible pathways of malic acid production by Gram negative bacteria, which is likely to influence phosphate solubilization. Therefore future work has to be initiated in this direction to explore the pathways of malic acid production in Gram negative bacteria and to further elucidate its role in bacterial phosphate solubilization.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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