Indian Journal of Biochemistry & Biophysics
Vol. 59, October 2022, pp. 947-955
DOI: 10.56042/ijbb.v59i10.67099

Comparative P solubilizing efficiencies of five acid soil bacteria incubated with calcium, aluminium and iron phosphates

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Received 06 March 2019; *revised 31 March 2022*

As acid soils of Odisha have been facing a major constraint in phosphorus availability, application of native P solubilizing bacteria could be promising as well as ecofriendly step towards sustainable P availability for crop growth and development. To address the problem of P availability in acid soil of Bhubaneswar, Odisha, rhizosphere soil samples (pH ≤ 5.50) with rice – pulses (green gram/black gram) cropping system were collected and phosphate solubilising bacteria were isolated. *In vitro* characterization of the PSB isolates were conducted with calcium, aluminium and iron phosphates to recover soluble P. All the five strains *i.e.Bacillus cereus* BLS18 (KT582541), *Bacillus amyloliquefaciens* CTC12 (KT633845), *Burkholderia cepacia* KHD08 (KT717633), *Burkholderia cepacia* KJR03 (KT717634), *Burkholderia cepacia* K1 (KM030037) could solubilize $Ca_3(PO_4)_2$, $AlPO_4$, $FePO_4$, and $Fe_3(PO_4)_2$. Higher recovery of soluble P was with $Ca_3(PO_4)_2$ while the least was with $AlPO_4$. All the strains exibited a trend similar with respect to P recovery *i.e.* $Ca_3(PO_4)_2$> $FePO_4$> $AlPO_4$> $Fe_3(PO_4)_2$. *B. amyloliquefaciens* CTC12 was most efficient in solubilizing calcium and iron phosphates whereas *B. cepacia* KHD08 recovered maximum P with aluminium phosphate. All the inorganic salt fortified mediums showed a significant decline in pH which necessitated the identification of compounds present in the mediums. Organic acids *viz*; acetic, citric, gluconic, lactic, malic, succinic, tartaric acids in the mediums were identified by HPLC. Tartaric acid was only found in the mediums supplemented with $AlPO_4$. *B. amyloliquefaciens* CTC12 and *B. cepacia* KHD08 showed promising results in *in vitro* analysis of P solubilization. The present study is focused on problematic acid soils where phosphorous is unavailable and mostly fixed with aluminium and iron ultimately making it unavailable for the crops to take up. This leads to unbalanced and frequent use of chemical fertilizer. Hence the study is a significant attempt to characterize native PSBs with capacity to solubilize Al-P and Fe-P.

Keywords: *Bacillus*, Biofertilizer, *Burkholderia*, Gluconic acid, HPLC

In a terrestrial ecosystem, soil microorganisms are playing an important role in the rhizosphere of plants involved in the recycling, convertion and solubilization of nutrients and crucial for long-term soil sustainability[1]. The use of chemical fertilizers appears as an extra burden on the farmers apart from causing environmental problems[2]. After nitrogen, phosphorus (P) is one of the most important chemical elements for plant growth and development as it exists as a part of the ATP molecules and plays a decisive role in the DNA chain[3]. Now a day's recent sustainable agricultural practices promote towards use of phosphate solubilizing bacteria (PSB), either in conjunction with or as a replacement for expensive and environmentally damaging fertilizers[4]. Acidic soils such as alfisols, aridisols, entisols and ultisols occupy about 30 per cent of the world's ice-free land area. In India acid soils extend over an area of 49 M ha (30 per cent of cultivated land) out of which 25 Mha have pH below 5.5 and 24 Mha between 5.5 and 6.5[5]. In Odisha acid soils occupy about 70 per cent of the total cultivated area (6.1 M ha)[6] extending over entire upland (46%) and major part (30%) of the medium lands. Around 21.2 per cent of the acid soil (pH >5.5) in Odisha is strongly acidic in nature.

Among the nutrients harmed due to soil acidity, phosphorous is most prominent. Many workers have reported about the effect of acidity on solubility of soil P. Decreased pH increases the anion exchange capacity (AEC) of soil, which in turn brings P fixation. In the soil environment, P in the phosphate form (PO_4^{2-}), invariably

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forms compounds with calcium, aluminium and iron, making it unavailable for crop uptake[7]. To counteract this behaviour of P a quite huge amount of phosphatic fertilizers are added to soil which quickly get fixed with calcium, aluminium and iron metallic ions making it unavailable for crop uptake.

Excessive application of phosphatic fertilizers to P deficient soil hampers soil chemical and biological environment. Soil microorganisms can be used as the alternative eco-friendly approach. Phosphate-solubilizing microorganisms possess the ability to transform soluble phosphates from its insoluble forms indirectly improving plant growth[8]. These microorganisms possess mineral phosphate solubilizing (MPS) ability with inorganic P sources *viz*; calcium phosphate tribasic [$Ca_3(PO_4)_2$], iron phosphate ($FePO_4$) and aluminium phosphate ($AlPO_4$)[9]. Potential strain of phosphatesolubilizing bacteria Klebsiella sp. from chromium contaminated agricultural soils identified for chromium (VI) remediation (Gupta *et al*., 2018). Microbes employ different mechanisms for transformation of unavailable P forms into bioavailable P forms through acidification, chelation, exchange reactions and secretion of organic acids[10]. However, heterotrophic microorganisms mostly secrete organic acids, which perform as chelating agent to bring the phosphate ion to the soil solution[11]. The organic acids, 2-keto gluconic and gluconic acid released by bacteria contribute the major part for inorganic/mineral phosphate solubilization[12].

At present, P solubilizing bacteria have been isolated from the rhizospheres of field crops[13], but there are few reports on isolation of PSBs from acid soils. In the present investigation, we have screened P solubilizing efficiencies of five strains of PSBs isolated from acid soils of Odisha and quantified the organic acids produced in *in vitro* cultures.

Materials and methods

Collection of soil samples and screening of P solubilizing bacteria

GPS based rhizospheric soil samples (0-15 cm depth) were collected from Mayurbhanj, Balasore, Cuttack, Khordha and Keonjhar districts of Odisha and analysed for soil reaction (pH) following standard method[14]. Soils with pH below 5.50 were selected for isolation of P solubilizing bacteria using National Botanical Research Institute's phosphate (NBRIP) growth medium[15] supplemented with tricalcium phosphate (TCP).

Evaluation of P solubilization efficiencies

Screened PSBs were further examined in NBRIP liquid medium fortified with inorganic phosphates [$Ca_3(PO_4)_2$, $AlPO_4$, $FePO_4$ and $Fe_3(PO_4)_2$]. The flasks were incubated at 30 ± 2°C for 72 h and the contents were centrifuged at 10,000 rpm for 30 min. Soluble free phosphate in culture supernatant was estimated from the absorbance values obtained using the calibration curve with KH_2PO_4 at 660 nm for each strain[14,16,17]. The potent strains were incubated till 7th day continually soluble P content was measured.

Organic acids secretion

Screened PSBs were further examined in NBRIP liquid medium added with inorganic phosphates [$Ca_3(PO_4)_2$, $AlPO_4$, $FePO_4$ and $Fe_3(PO_4)_2$]. The flasks were incubated at 30 ± 2^0 C for 8 days and the contents were centrifuged at 10,000 rpm for 30 min. The supernatants were analysed in HPLC for detection of organic acids by comparing with the pure organic acid standards[18].

Instrument parameters

All the samples and solutions of the mobile phase were filtered through a 0.25 μm membrane filter (cellulose acetate) prior to injecting into the Chromatograph column. The C18 chromatograph column was selected (LiChrospher® 100 RP-18 (5 μM), Merck Millipore). Analysis was done in HPLC-PDA system (LC-20AT, Shimadzu Corporation, Tokyo, Japan). As mobile phase, 0.1%TFA at pH 3.0 in HPLC grade water + HPLC grade methanol (95:5, V/V) was used at a flow rate of 0.8 mL min^{-1}. Organic acids peaks were detected at a wavelength of 210 nm with photodiode array (PDA) detector. Individual organic acids were identified and calibrated by comparing retention times with those of the standards prepared with known amounts of TA, GA, MA, LA, AA, CA, SA that eluted with a retention time 3.25, 3.60, 4.90, 5.70, 6.48, 8.20, 8.80 min, respectively.

Statistical Analysis

Statistical analysis was performed by using software R version 3.2.2 and were tested with Duncan's new multiple range test at 5% critical range using the package "agricolae". The values are the means of four replicates.

Results

P solubilizing bacteria from acid soils of Odisha

Out of 30 districts only five (5) selected districts *viz*; Balasore, Cuttack, Khordha, Keonjhar and Mayurbhanj with around 41.5, 96.5, 82.3, 30.9 and 81.0 per cent acid soil areas, respectively, were sampled. GPS based rhizosphere soil samples (250 nos.) were collected

from these five districts and out of 250 samples only 106 nos. with pH ≤ 5.50 were selected for enumeration of phosphate solubilizing bacteria. A total of two hundred eight (208) nos. of PSB isolates were isolated from the villages of five districts. Forty five (45) PSB isolates with ≥ 180% P solubilization efficiency on NBRIP agar medium with tricalcium phosphate as the P source were selected for further study. Basing on the soluble P recovery by the 45 PSB isolates at 48 and 72 h of incubation; five (5) isolates *viz*; BLS18 (Sarupala, Balasore), CTC12 (Echhapur, Cuttack), KHD08 (Balarampur, Khordha), KJR03 (Rangadihi, Keonjhar) and K1 (Chitrada, Mayurbhanj), one from each district were selected for further screening and characterization of their P solubilizing efficiency. Following biochemical, 16srDNA characterization and the dendrogram (data not presented) the isolates BLS18, CTC12, KHD08, KJR03, K1 were identified as strains of *Bacillus cereus* (KT582541), *Bacillus amyloliquefaciens*(KT633845), *Burkholderia cepacia* (KT717633), *Burkholderia cepacia* (KT717634), *Burkholderia cepacia* (KM030037), respectively.

Soluble P recovery with different inorganic P sources at 72 and 168 h incubation

The five PSB strains were incubated in NBRIP broth medium supplemented with $Ca_3(PO_4)_2$, $AlPO_4$, $FePO_4$ and $Fe_3(PO_4)_2$. The quantities of soluble phosphorous (P) in broths were measured at 72, and 168 h (Table 1). Among the P supplemented sources, maximum soluble P recovery was in $Ca_3(PO_4)_2$ followed by $FePO_4$, $Fe_3(PO_4)_2$ and $AlPO_4$ in declining order. At 72 h of incubation *Bacillus amyloliquefaciens* CTC12 recovered maximum soluble P (327.34 mg/L) and KJR03 the least (156.85 mg/L) in the $Ca_3(PO_4)_2$ supplemented medium. In the medium with Al-P, *Burkholderia cepacia* KHD08 solubilized maximum P (20.05 mg/L) followed by CTC12 (18.80 mg/L). *Bacillus amyloliquefaciens* CTC12 solubilized maximum P *i.e*. 84.00 and 21.00 mg/L, respectively, in Fe(III)-P and Fe(II)-P mediums. After a period of 168 h of incubation *Bacillus amyloliquefaciens* CTC12 continue to recover maximum soluble P *i.e*. 473.25, 199.00 and 75.00 mg/L, respectively, in Ca-P, Fe(III)-P and Fe(II)-P mediums whereas *Burkholderia cepacia* KHD08 showed maximum P solubilization (58.49 mg/L) with Al-P as insoluble P source. Continuing the incubation further till 192 h, similar trend was observed. After the 8th day, *Burkholderia cepacia* K1 solubilized least P (367.75 mg/L) in Ca-P supplemented medium whereas, *Bacillus cereus*BLS18 (31.75 mg/L) and (88.10 mg/L) in Al-P and Fe(II)-P medium, respectively, whereas *Burkholderia cepacia* KJR03(45.25 mg/L) in Fe(III)-P.

Relationship between soluble P and pH of the cultured supernatant

Data presented in Figure 1A-G depicted that the pH of the cultured supernatants ranged between 3.53 to 4.73 at 72 h and 3.50 to 4.60 at 168 h irrespective of the bacterial isolates used. A negative correlation was observed between soluble P and pH of the culture medium till 192 h of incubation. At 72 h of incubation soluble P and pH of the cultured supernatant exhibited inverse correlation with R^2 values of 0.882, 0.880, 0.832 and 0.972, respectively, for mediums supplemented with $Ca_3(PO_4)_2$, $AlPO_4$, $FePO_4$ and $Fe_3(PO_4)_2$. Similar inverse correlation was also observed at 168 h of incubation between soluble P and pH of the cultured supernatant.

Organic acid secretion

After 8 days of incubation, organic acids *viz*; acetic, citric, gluconic, lactic, malic, succinic, tartaric acids were detected in the undiluted cultured supernatant of the five PSB isolates and presented in the (Table 2 and Figs 2-5). The produced organic acids were identified by comparing their retention

Table 1 — Soluble phosphorous recovery by the five PSB strains with different inorganic P sources

Isolates	Soluble P (mg/L)							
	72 h incubation				168 h incubation			
	$Ca_3(PO_4)_2$	$AlPO_4$	$FePO_4$	$Fe_3(PO_4)_2$	$Ca_3(PO_4)_2$	$AlPO_4$	$FePO_4$	$Fe_3(PO_4)_2$
BLS18	185.00±3.06^{b}	9.50±0.052^{b}	45.83±2.007^{b}	6.05±0.672^{b}	337.75±0.624^{c}	25.61±0.430^{c}	95.00±0.451^{b}	38.67±0.287^{b}
CTC12	327.34±4.78^{a}	18.80±0.064^{a}	84.00±3.188^{a}	21.00±1.954^{a}	473.25±0.515^{a}	34.50±0.722^{bc}	199.00±0.374^{a}	75.00±0.335^{a}
KHD08	209.25±3.05^{b}	20.05±0.035^{a}	47.15±1.836^{b}	17.49±0.794^{a}	433.75±1.317^{ab}	58.49±0.430^{a}	149.30±0.735^{ab}	67.01±1.084^{a}
KJR03	156.85±2.83^{b}	7.15±0.035^{b}	42.50±1.763^{b}	7.15±0.174^{b}	388.20±1.198^{bc}	48.90±0.487^{ab}	83.50±0.762^{b}	34.75±0.622^{b}
K1	201.75±3.42^{b}	6.50±0.058^{b}	39.20±2.014^{b}	13.30±1.635^{ab}	349.75±0.919^{c}	29.89±0.274^{bc}	89.00±0.899^{b}	53.50±0.660^{ab}

Tested by Duncan's Multiple Range Test with 5% critical range. Means represented by the same letter are not significantly different. Data given in above are average values of four replicates ± standard error of mean (SEM)

times with standard samples. Results revealed higher volume of tartaric acid in the medium supplemented with $AlPO_4$as inorganic P source. *Bacillus cereus*BLS18 produced more volume of tartaric acid (69.99 mM) in the medium with $AlPO_4$ followed by *Burkholderia cepacia* KHD08 (61.54 mM). Lowest quantity of tartaric acid (0.11 mM) was produced only by *Bacillus cereus*BLS18 in the medium with $Fe_3(PO_4)_2$. Lactic acids of quantity 12.83, 18.26 mM, respectively, was produced by *Bacillus cereus* BLS18 in medium with $Ca_3(PO_4)_2$ and $Fe_3(PO_4)_2$ as insoluble P sources. *Bacillus cereus* BLS18 also produced small amounts of malic acid (2.00 mM) in the medium with $Ca_3(PO_4)_2$. *Bacillus amyloliquefaciens* CTC12 produced acetic acid (15.75 mM), citric acid (0.15 mM), lactic acid (2.85 mM), succinic acid (3.91 mM) and tartaric acid (4.73 mM) in the medium with $Ca_3(PO_4)_2$ as inorganic phosphate. *Bacillus amyloliquefaciens* CTC12 also produced acetic acid (8.55, 4.56 mM), lactic acid (1.37, 48.17 mM), malic acid (0.68, 0.30 mM), tartaric acid (2.57, 2.32 mM), citric acid (2.32 mM) and succinic acid (5.33 mM), respectively, in mediums with $FePO_4$ and $Fe_3(PO_4)_2$ as insoluble P sources but could produce only tartaric acid (54.56 mM) in $AlPO_4$ medium. Three strains (KHD08, KJR03 and K1) of *Burkholderia cepacia* yielded gluconic acid of quantities 36.69, 15.19 and 12.55 mM, respectively, in the $Ca_3(PO_4)_2$ supplemented medium. *Burkholderia cepacia* KHD08 could release only tartaric acid (61.54 mM) in the $AlPO_4$ medium. Similarly two other strains (KJR03 and K1) of *Burkholderia cepacia* KJR03 and K1, respectively, resulted in 61.13 and 50.24 mM of tartaric acid production in $AlPO_4$ medium.

Discussion

One of the major drawbacks of acid soil is P fixation. It fixes the orthophosphate form of P into calcium,

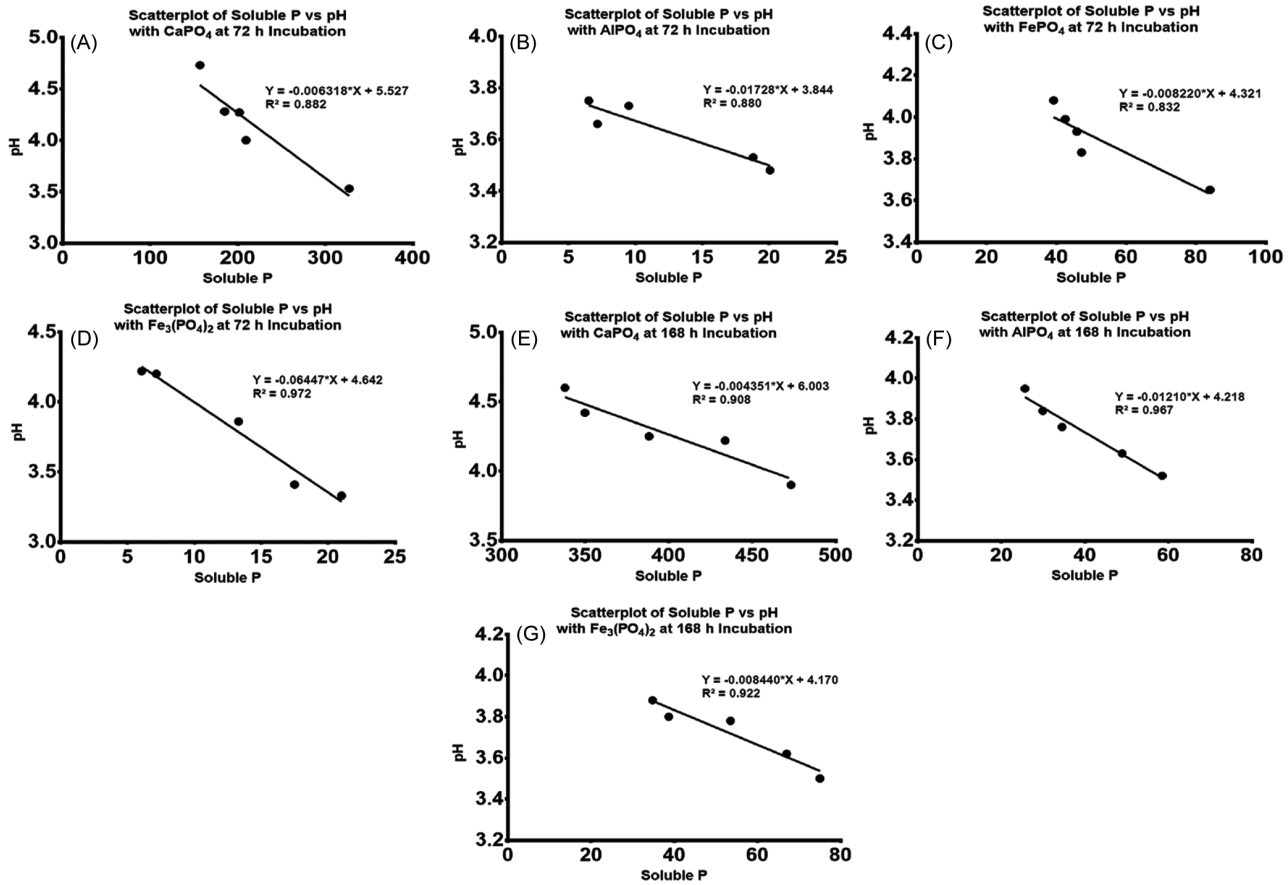

Fig. 1 — (A) Correlation between soluble P and pH of NBRIP broth ($Ca_3(PO_4)_2$) over 72 h of incubation; (B) Correlation between soluble P and pH of NBRIP broth ($AlPO_4$) over 72 h of incubation; (C) Correlation between soluble P and pH of NBRIP broth ($FePO_4$) over 72 h of incubation; (D) Correlation between soluble P and pH of NBRIP broth ($Fe_3(PO_4)_2$) over 72 h of incubation; (E) Correlation between soluble P and pH of NBRIP broth ($Ca_3(PO_4)_2$) over 168 h of incubation; (F) Correlation between soluble P and pH of NBRIP broth ($AlPO_4$) over 168 h of incubation; and (G) Correlation between soluble P and pH of NBRIP broth ($Fe_3(PO_4)_2$) over 168 h of incubation

aluminum and iron phosphates. In acidic soils these aluminum and iron phosphate forms are more prevalent. In the present context, we have targeted five (5) districts *i.e.*Balasore (extent of soil acidity-41.5%), Cuttack (96.5%), Khordha (82.3%), Keonjhar (30.9%) and Mayurbhanj (81.0%). A total of 250 nos. of GPS based rhizospheric soil samples collected from different villages and blocks of these districts of Odisha were subjected for analysis of soil reaction (pH). Out of which 106 nos. of soil samples with pH

Table 2 — Organic acids production by the five PSB strains

Isolates	P Sources	Organic acids (mM)						
		AA	CA	GA	LA	MA	SA	TA
BLS18	$Ca_3(PO_4)_2$	-	-	-	12.83	2.00	-	0.33
	$AlPO_4$	-	-	-	-	-	-	69.99
	$FePO_4$	0.52	-	-	0.32	-	-	-
	$Fe_3(PO_4)_2$	-	-	-	18.26	-	3.25	0.11
CTC12	$Ca_3(PO_4)_2$	15.75	0.15	-	2.85	-	3.91	4.73
	$AlPO_4$	-	-	-	-	-	-	54.56
	$FePO_4$	8.55	-	-	1.37	0.68	-	2.57
	$Fe_3(PO_4)_2$	4.56	2.32	-	48.17	0.30	5.33	2.32
KHD08	$Ca_3(PO_4)_2$	14.44	-	36.69	3.99	-	2.65	-
	$AlPO_4$	-	-	-	-	-	-	61.54
	$FePO_4$	1.62	0.33	0.54	38.88	-	0.24	4.73
	$Fe_3(PO_4)_2$	-	-	0.23	4.97	-	-	2.56
KJR03	$Ca_3(PO_4)_2$	4.15	1.27	15.19	0.55	-	-	2.63
	$AlPO_4$	-	-	-	-	-	-	61.13
	$FePO_4$	-	-	0.79	0.26	-	0.66	-
	$Fe_3(PO_4)_2$	0.56	0.28	0.45	1.44	-	1.88	1.47
K1	$Ca_3(PO_4)_2$	-	-	12.55	22.05	0.99	0.92	0.30
	$AlPO_4$	-	-	-	-	-	-	50.24
	$FePO_4$	0.17	-	0.88	0.96	-	-	-
	$Fe_3(PO_4)_2$	1.22	-	0.45	3.09	-	-	-

*AA- Acetic Acid, CA- Citric Acid, GA- Gluconic Acid, LA- Lactic Acid, MA- Malic Acid, SA- Succinic Acid, TA- Tartaric Acid

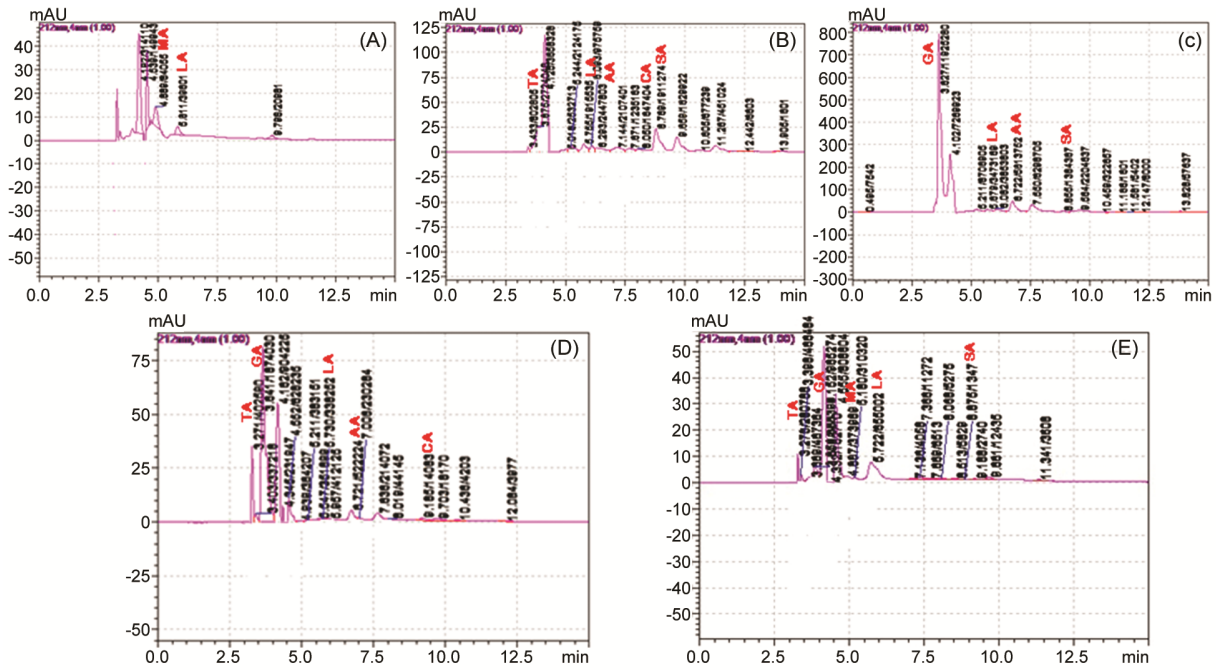

Fig. 2 — HPLC analysis of the known (AA, CA, GA, LA, MA, SA, TA) and unknown organic acids detected in the cultured supernatant of the five PSB isolates [A. BLS18, B. CTC12, C. KHD08, D. KJR03 and E. K1] in the NBRIP medium [$Ca_3(PO_4)_2$]

≤ 5.50 were selected for enumeration of mineral or inorganic P solubilizing bacteria. The state receives broad range variations with respect to climate, geology, rainfall, land forms and vegetation resulting in different variety of soil forms[19].

A total of two hundred eight (208) PSB isolates were screened in which Mayurbhanj district ranked the top in the no. of PSB isolates followed by Balasore and Cuttack district. Basing on their P solubilization efficiency (≥180.00%) on NBRIP agar medium with

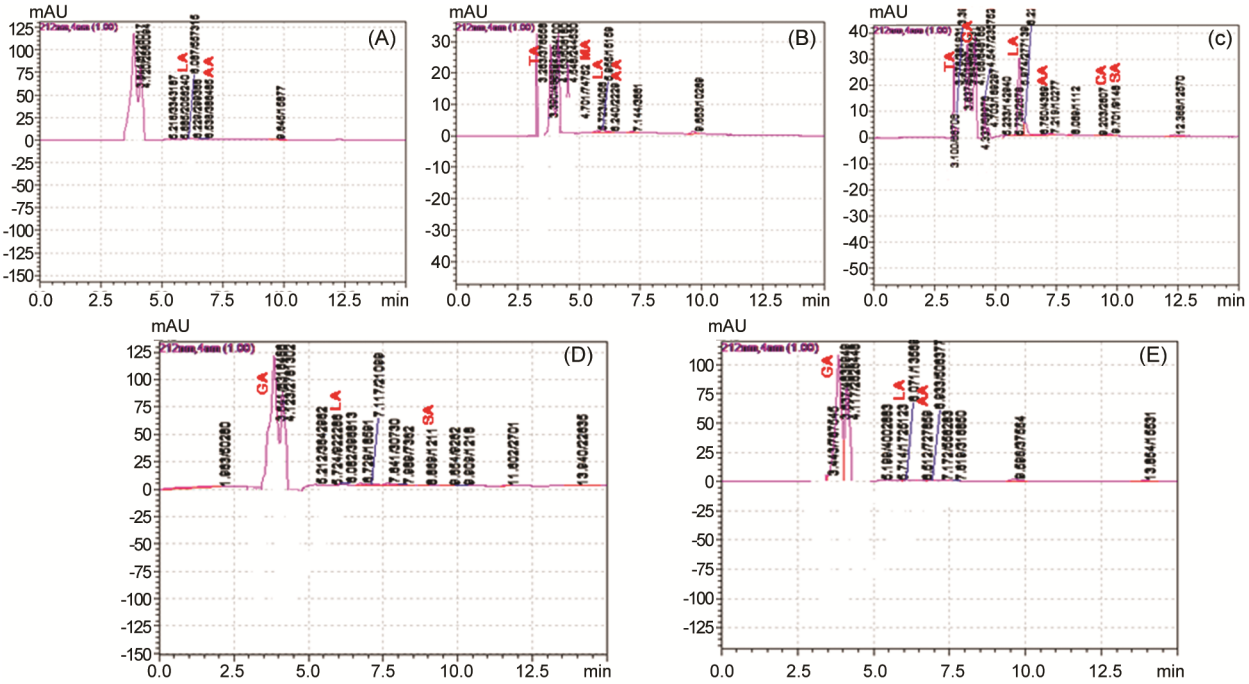

Fig. 3 — HPLC analysis of the known (TA) and unknown organic acids detected in the cultured supernatant of the five PSB isolates [A. BLS18, B. CTC12, C. KHD08, D. KJR03 and E. K1] in the NBRIP medium [$AlPO_4$]

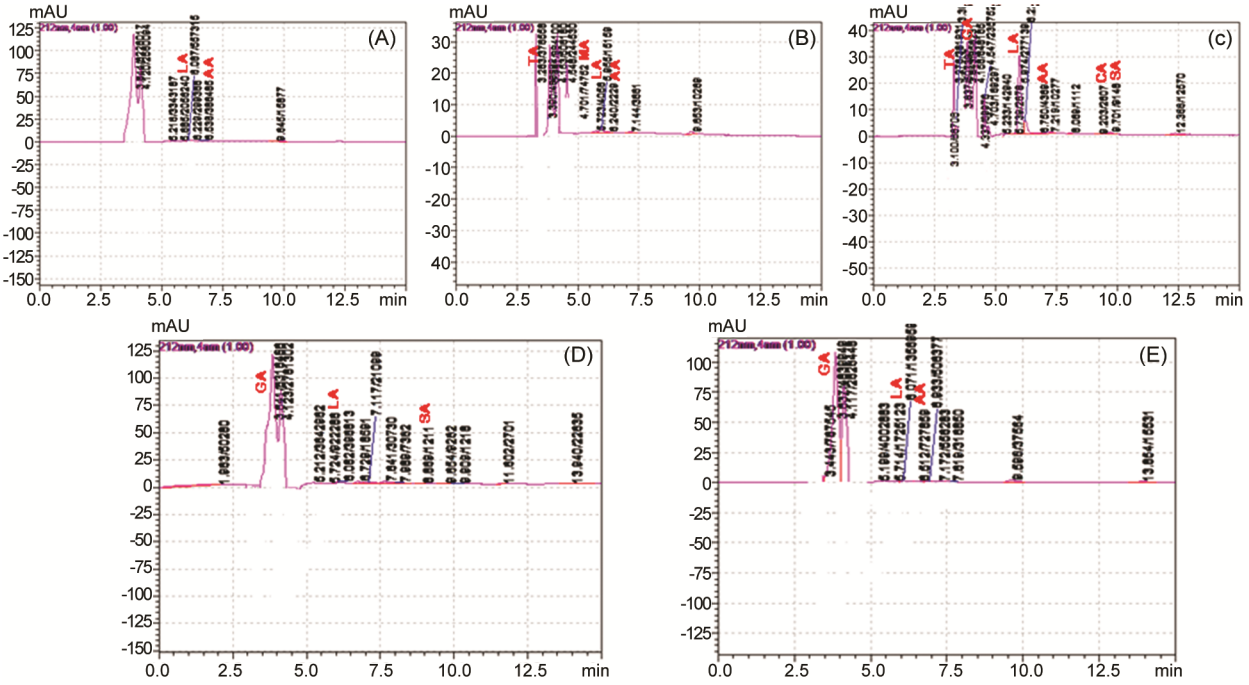

Fig. 4 — HPLC analysis of the known (AA, CA, GA, LA, MA, SA, TA) and unknown organic acids detected in the cultured supernatant of the five PSB isolates [A. BLS18, B. CTC12, C. KHD08, D. KJR03 and E. K1] in the NBRIP medium [$FePO_4$]

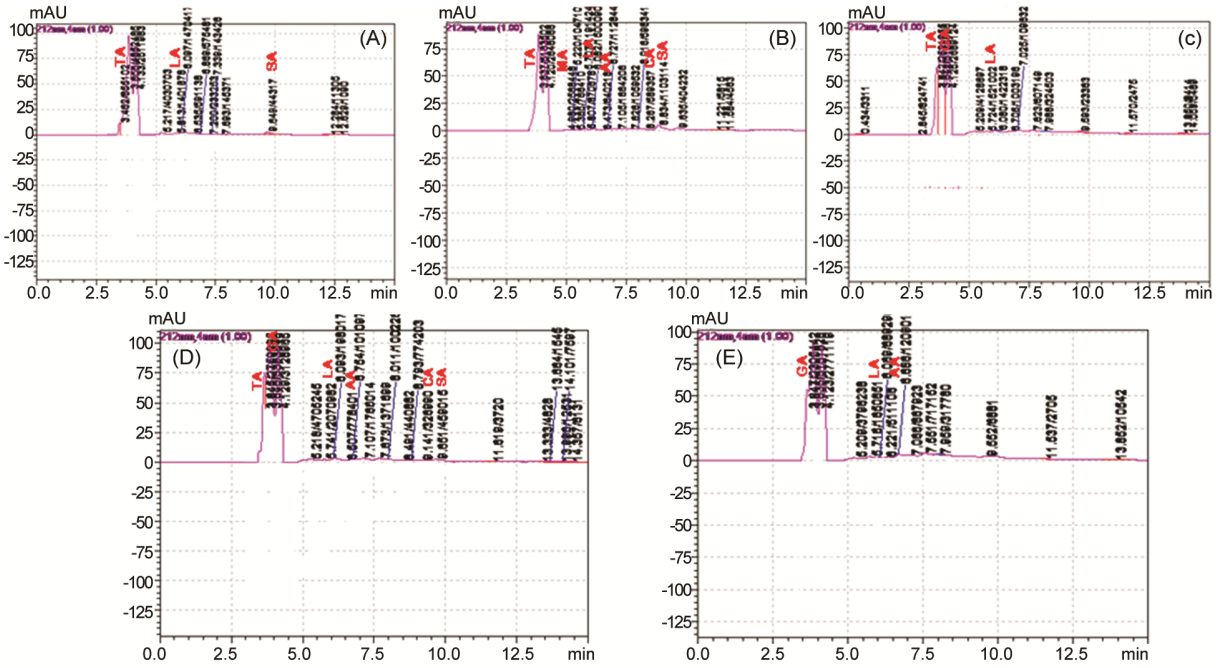

Fig. 5 — HPLC analysis of the known (AA, CA, GA, LA, MA, SA, TA) and unknown organic acids detected in the cultured supernatant of the five PSB isolates [A. BLS18, B. CTC12, C. KHD08, D. KJR03 and E. K1] in the NBRIP medium [$Fe_3(PO_4)_2$]

tricalcium phosphate as the P source[15], they are again screened to forty five (45) numbers.

To maintain nativity of the strain, we have selected one isolate from each district with highest P solubilization potency and optimum P recovery with the given inorganic P sources. The isolates were BLS18 (Balasore - N21^{0}30.666', E86^{0}47.575'), CTC12 (Cuttack - N20^{0}31.105', E85^{0}33.624'), KHD08 (Khordha - N20^{0}11.881', E85^{0}27.827'), KJR03 (Keonjhar - N21^{0}36.787', E85^{0}27.219') and K1 (Mayurbhanj - 21^{0}50.487', 86^{0}56.986'). BLS18 and CTC12 were gram positive rods and the rest three were gram negative rods. The isolates were identified as strains of *Bacillus cereus* BLS18 (KT582541), *Bacillus amyloliquefaciens* CTC12 (KT633845), *Burkholderia cepacia* KHD08 (KT717633), *Burkholderia cepacia* KJR03 (KT717634), *Burkholderia cepacia* K1 (KM030037). Earlier workers have stated that, variety of microflora (*Achromobacter, Agrobacterium, Bacillus, Enterobacter, Erwinia, Escherichia, Flavobacterium, Mycobacterium, Pseudomonas* and *Serratia*) either gram positive or negative could efficiently solubilized the inorganic phosphorus from its unavailable sources[20-22].

Since NBRIP (National Botanical Research Institute Phosphate) growth medium with tricalcium phosphate as the insoluble P source should not be considered as the sole selector for isolation of efficient P- solubilizers[23] and as we targeted only acid soils, which predominantly contains $AlPO_4$, $FePO_4$ and $Fe_3(PO_4)$[23,24]as the inorganic phosphate complexes, hence NBRIP broth mediums were used with aluminium and iron as inorganic phosphates. These P compounds are stable minerals with very poor solubility which directly hinder the P uptake by plants. P in the forms of orthophosphates ($H_2PO_4^-$ and HPO_4^{-2}) can only be taken up by the plants[23].

The soluble P recovery by all the isolates with different P sources followed the order $Ca_3(PO_4)_2$> $FePO_4$> $AlPO_4$> $Fe_3(PO_4)_2$. Further, there was an increasing trend in the soluble P recovery with increase in incubation period. Thus, after 168 h incubation more phosphorus was recovered compared to 72 h by all the 45 PSB isolates irrespective of the P sources supplied. Chung *et al*. (2005) also reported 13 best isolates based on the solubilization of insoluble phosphates ($Ca_3(PO_4)_2$, $FePO_4$ and $AlPO_4$) in liquid culture. However, the cultured supernatant when subjected to analysis of pH at 48 and 72 h, all the culture mediums showed a decline in pH *i.e*. below 5.30, which implied direct linkage of P-solubilization with reaction (pH) of the cultured broth. Higher the soluble P, lower is the pH of the medium. One of the best understood mechanisms of P solubilization is the secretion of various low molecular weight organic acids *viz*; succinic, oxalic, malic, propionic, gluconic, 2-ketogluconic, citric,

acetic, isovaleric, heptanoic, caproic, formic, n-butyric, oxalic, methylmalonic acids[18,25,26]. Decrease in pH of the medium at 48 and 72 h incubation indicated production of organic acids resulting in mineral P solubilization[27]. At 7th day more soluble phosphates were recovered compared to the observation made on the 3rd day. More particularly KHD08, KJR03 recovered significantly similar amounts of soluble phosphates from $AlPO_4$ after 168 h. But in case of $FePO_4$ no significant difference was obtained with the inoculation of strains BLS18, KJR03 and K1 at 168 h. Strains CTC12 and KHD08 recovered significantly higher amounts of soluble P after 168 h of incubation. However, in case of $Fe_3(PO_4)_2$ the PSB strains CTC12, KHD08 and K1 were found more efficient in solubilizing phosphates after 168 h of incubation. Microorganisms possess the ability of mineral P solubilization with hard to dissolve complexes like Al-P and Fe-P[28,29]. It has been reported that structural complexities of different mineral phosphates affect the phosphate solubilization efficiencies of different PSB strains[30].

HPLC analysis of the cultured supernatant after 8th day of incubation revealed the presence of low molecular weight organic acids (acetic, citric, gluconic, lactic, malic, succinic and tartaric acids) in all the mediums. The type of P source had an effect on the type and amount of organic acids excreted into the respective growth medium. All the isolates produced one or more organic acids in all the mediums[31, 32]. Only tartaric acid was found in the mediums supplemented with $AlPO_4$ as the P source irrespective of the PSB isolates inoculated in the mediums. The threegram negative PSB isolates secreted gluconic acids in all the mediums except the medium with $AlPO_4$. No gluconic acids were found with the two gram positive isolates *viz*; BLS18 and CTC12[33, 34]. Some of the unknown peaks were also observed in the HPLC analysis which couldn't be recognized. The P-solubilizing activity is determined by the ability of the microbes to release metabolites such as organic acids, which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, the latter being converted to soluble forms. Production and release of organic acids is the primary mechanism involved in inorganic P solubilization[34]. In most bacteria, mineral phosphate-dissolving capacity has been shown to be related to the production of organic acid. The levels of gluconic and 2-keto gluconic acids are more in the soils adjacent to the plant roots where the availability of glucose would be higher than in the bulk soils. Results of the study confirmed that mineral P solubilization by different PSB isolates require essentially the involvement of organic acids [32,35]. Identification and quantification of organic acids established the inverse relationship between pH of the medium and mineral P solubilization[18,36].

Conclusion

Five isolates screened for acidic soil recovered soluble P when incubated with calcium, aluminium and iron phosphates and followed the order $Ca_3(PO_4)_2$> $FePO_4$> $AlPO_4$> $Fe_3(PO_4)_2$. *Bacillus amyloliquefaciens*CTC12 recovered highest amounts of soluble P *in vitro* when incubated with $Ca_3(PO_4)_2$, $FePO_4$ and $Fe_3(PO_4)_2$ whereas *Burkholderia cepacia* KHD08 recovered maximum available P with $AlPO_4$. Organic acids *viz*; acetic, citric, gluconic, lactic, malic, succinic, tartaric acids were detected in the cultured supernatant of all the isolates irrespective of the P source. Only tartaric acid was found in the medium supplemented with $AlPO_4$ as the P source irrespective of the PSB strains. The three gram negative PSB strains (KHD08, KJR03 and K1) of *Burkholderia cepacia* secreted gluconic acids in all the mediums except in the medium with $AlPO_4$. However, no gluconic acids were found with the two gram positive bacteria *i.e. Bacillus cereus* BLS18 and *Bacillus amyloliquefaciens* CTC12. Though the *in vitro* P solubilizing traits of these bacteria were satisfactory enough to denote them as P solubilizing bacteria, but field efficiency study is needed to uncover their efficacy as biofertilizer.

Acknowledgement

Authors would like to thank Steel Authority of India Ltd. (SAIL), Rourkela, India for the funding to accomplish the research work. Authors would also like to acknowledge COE, World Bank, Utkal University, Bhubaneswar, Odisha.

Conflict of interest

All authors declare no conflict of interest.

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