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Short communication

Gluconic acid production as the principal mechanism of mineral phosphate solubilization by *Burkholderia* sp. (MTCC 8369)

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Abstract

Eighty one potential phosphate solubilizing bacteria (PSB) were isolated from the rhizosphere soils of crop plants in Athirampuzha, Kottayam, Kerala and were screened for their efficiency. *Burkholderia* sp. (MTCC 8369) emerged as the most efficient isolate based on biochemical characteristics, FAME analysis, and 16S rDNA typing (GQ246871). The conditions for maximum P solubilisation were: pH 7, temperature 32.5ºC, 18 days of incubation, glucose as a source of carbon, ammonium sulphate as N source, 0.5–1% salt, and 3% sugar. HPLC analysis of the culture filtrate identified gluconic acid as the principal organic acid released by *Burkholderia* sp. The isolate also exhibited antifungal activity and produced indole acetic acid (IAA), siderophore, hydrogen cyanide, and exopolysacharides. This isolate with its multidimensional plant growth promotion characteristics broadens the spectrum of phosphate solubilizers for field application.

Keywords: Plant growth-promoting bacteria, 16S rDNA typing, FAME Analysis, Siderophore.

Phosphorus (P) is one of the most essential macro-nutrients required for harnessing the yield potential of high yielding varieties in intensive and multiple cropping systems. Chemical P fertilizers added to the soils to circumvent the problem of P deficiency, however, are precipitated (75 to 90%) by Fe, Al, and Ca complexes present in the soils (Gyaneshwar et al., 2002), compounding the problem. Many soil micro-organisms mobilize insoluble inorganic phosphates from their mineral matrix to the soil solution, making them available to plant roots. Occurrence of phosphate solubilizing microorganisms has been reported from different environmental niches (Chen et al., 2006). There are also several reports regarding plant growth promotion due to inoculation of phosphate solubilising microorganisms under greenhouse as well as field conditions emphasizing the need for evolving efficient strains (Gyaneshwar et al., 2002). In recent decades, increasing evidences indicate that besides increased nutrient uptake, the synthesis and export of phyto-hormones by micro-organisms may play an important role in plant growth promotion (Poonguzhali et al., 2008).

Although the mechanism of phosphate solubilization is still not clearly understood, production of organic acids seems to be important. Low molecular weight organic acids, mainly gluconic and ketogluconic acids are responsible for tricalcium phosphate and rock phosphate solubilization (Kumari et al., 2008). Soils of Kerala are highly P fixing and the P solubilizing organisms have a significant role (Sivaprasad and Meenakumari, 2005). An attempt was made to isolate and screen potent indigenous phosphate solubilizing bacteria (PSB) from the rhizosphere soils of major crop plants and to study gluconic acid production as the mechanism of phosphate solubilization.

Isolation of phosphate solubilizing bacteria was carried out from the rhizosphere soils of black pepper (*Piper nigrum*), paddy (*Oryza sativa*), banana (*Musa* spp.),

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cowpea (*Vigna unguiculata*), ladies finger (*Abelmoschus esculentus*), pineapple (*Ananas comosus*), and rubber (*Hevea brasiliensis*), important crops of Athirampuzha, Kottayam (9°35'0"N; 76°31'0"E), Kerala, during 2008–2009. Pikovaskaya's medium was used for isolation, cultivation, and maintenance of PSB. Soluble P in the supernatant solution was determined by vanadomolybdate phosphoric yellow colour method. After preliminary screening, promising isolates (17) were re-evaluated for their phosphate solubilization potential using Pikovskay's and National Botanical Research Institute's Phosphate (NBRIP) medium containing bromophenol blue (BPB) formulations (Mehta and Nautiyal, 2001).

The selected strains were subjected to cultural, morphological, and biochemical characterization as mentioned in Bergey's Manual of Determinative Bacteriology. 16SrDNA sequencing was done and the sequences were analysed using the gapped BLASTn (www. ncbi.nlm. nig.gov) search algorithm. The sequence was deposited in the NCBI gene bank database. Selected isolates were also identified based on whole cellular fatty acids, methylated to their fatty acid methyl ester (FAME) profiles using the MDI system (Microbial Identification System, Inc., Newark, DE, USA) (De Freitas et al., 1997).

Impact of different carbon (C; glucose, lactose, sucrose, mannitol, galactose, arabinose, xylose, fructose) and nitrogen (N) sources (ammonium sulphate, asparagine, urea, ammonium nitrate, potassium nitrate, calcium nitrate) on phosphate solubilization by selected isolate was studied by replacing the existing C and N sources of Pikovaskaya's medium. Similarly, a range of pH (6, 6.5, 7, 7.25, 7.5, and 8), temperature (20, 25, 27.5, 30.0, 32.5, and 35ºC), salt (w/v- 0.1, 0.25, 0.5, 0.75, and 1 g. 100 mL^{-1}), and sugar (1, 2, 3, 4, 5, and 10 g. 100 mL^{-1}) regimes were evaluated. Quantitative estimation of P solubilisation was done at different incubation periods from 0 to 30 days. Organic acids were analyzed by LaChrom Merck Hitachi high performance liquid chromatography (HPLC) using Waters C18 column (4.6 x 250 mm, Waters 717 plus Autosampler, Mobile phase: 0.1% H_3PO_4 in water, F/Rate: 1.0 ml/min, Waters 2487 Dual Wavelength Absorbance Detector, 210 nm, inj. vol: 20 µl). Organic acids were identified by comparing with standards. Dual culture assay was performed to detect the antifungal activity of selected strains against phytopathogenic fungi (*Phytophthora* sp. and *Fusarium oxysporum*). Plant growth promoting traits of organisms were also evaluated using standard protocols. Statistical analysis was performed by Analysis of Variance (ANOVA), and the means were separated using Duncan's Multiple Range Test (DMRT).

Eighty one potential phosphate solubilizing bacteria were isolated from the rhizosphere soils of different crop plants of Athrampuzha, Kottayam of which 17 were promising showing an extent of solubilisation between 11.38 to 68.80 mg/100 ml of soluble P in liquid medium. Isolate PSB 73 which solubilised 68.80 mg/100 ml of soluble P in liquid medium was particularly promising (Table 1). It produced circular, pale brownish opaque colonies (1 x 2 µm in size), and was aerobic, Gram negative, nonsporulating with motile rods. PSB 73 was identified as *Burkholderia* sp. (MTCC 8369). The MIDI system identified PSB 73 as *Burkholderia gladioli* (SIM $\geq$ 0.361). Results of BLAST search of 16S rDNA sequences of PSB 73 re-confirmed that this organism belonged to the genus *Burkholderia* (Fig. 1). The sequence of the strain deposited in the NCBI Gene Bank was assigned with the accession number GQ246871.

Optimum pH, temperature, and salt concentration for this organism were: 7, 32.5ºC, and 0.5 to 1g 100 mL^{-1}, respectively. Phosphate solubilisation increased with increasing amounts of glucose up to 3% (w/v), but decreased thereafter, which is consistent with earlier observations (Son et al., 2006). Peak solubilisation (30.44 mg/100 ml of P) occurred after 18 days of incubation and decreased thereafter (Fig. 2), which is expected in view of the decrease in soluble P and the increase in pH of the medium caused by utilization of P and organic acids for PSBs own metabolism (Tripura et al. 2007). The isolate also showed maximum P solubilizing activity in the presence of glucose and ammonium sulphate, as reported by Son et al. (2006).

HPLC analysis revealed one major peak identified as gluconic acid (Fig. 3). Gluconic acid is one of the

Table 1. Secondary screening of phosphate solubilizing bacteria isolated from rhizosphere soils of crop plants in Athirampuzha, Kottayam, India.

Phosphate solubilising bacteria	pH	P solubilised (mg/100 ml)*	Solubilization zone dia, Z (mm)	Spot diameter, C (mm)	Phosphate solubilization efficiency[a] (SE%) = (Z–C)/C x 100
PSB 12	3.69	62.97	35	6	483
PSB 13	3.26	50.92	27	4	575
PSB 20	4.46	43.25	28	5	460
PSB 21	5.29	49.52	22	4	450
PSB 22	3.61	49.24	19	6	217
PSB 23	3.93	40.24	17	7	143
PSB 30	3.69	40.24	19	6	217
PSB 31	4.67	22.19	20	8	150
PSB 38	4.04	42.13	16	8	100
PSB 40	5.85	23.95	–	–	–
PSB 47	4.22	32.93	12	6	100
PSB 55	4.14	37.48	18	8	125
PSB 56	5.01	11.38	–	–	–
PSB 58	3.56	59.08	18	5	260
PSB 66	3.27	55.71	20	5	300
PSB 67	3.77	63.84	19	3	533
PSB 73	4.31	68.80	21	5	320

*Values are mean of three replications and deduced from the control value; [a]P solubilization efficiency in NBRIP agar.

Pair-wise distance

Organisms	1	2	3	4
Pseudomonas antimicrobica (AB021384)	0.000			
Burkholderia sp. (GQ246871)	0.009	0.000		
Burkholderia vandii (U96932)	0.011	0.002	0.000	
Burkholderia plantarii (U96933)	0.010	0.004	0.005	0.000

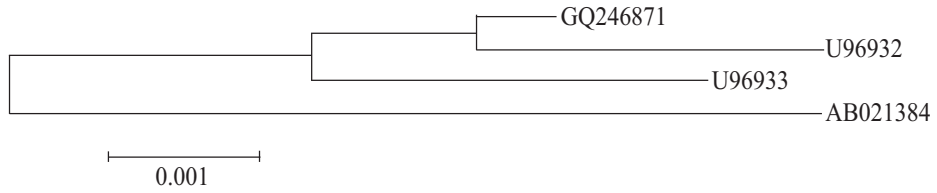

Figure 1. Phylogenetic tree based on 16S rDNA sequences drawn using the neighbour joining method. The tree was constructed by using the MEGA 3.1 after aligning the sequence with CLUSTALW and generating evolutionary distance matrix inferred by the neighbour joining method using Kimura parameter 2. The scale bar indicates 0.001 substitutions per nucleotide position.

prominent organic acids responsible for P solubilization and is produced by direct oxidation of glucose via membrane bound quinoprotein GDH enzyme. The resulting pH change and reduction potential are thought to be responsible for the dissolution of tricalcium phosphate in the culture medium (Chen et. al., 2006).

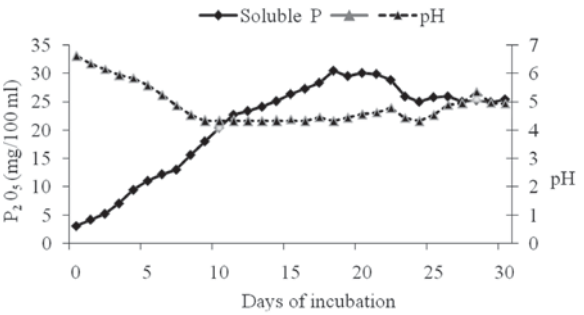

Figure 2. Rock phosphate solubilization and pH profile of *Burkholderia* sp. at different stages of incubation

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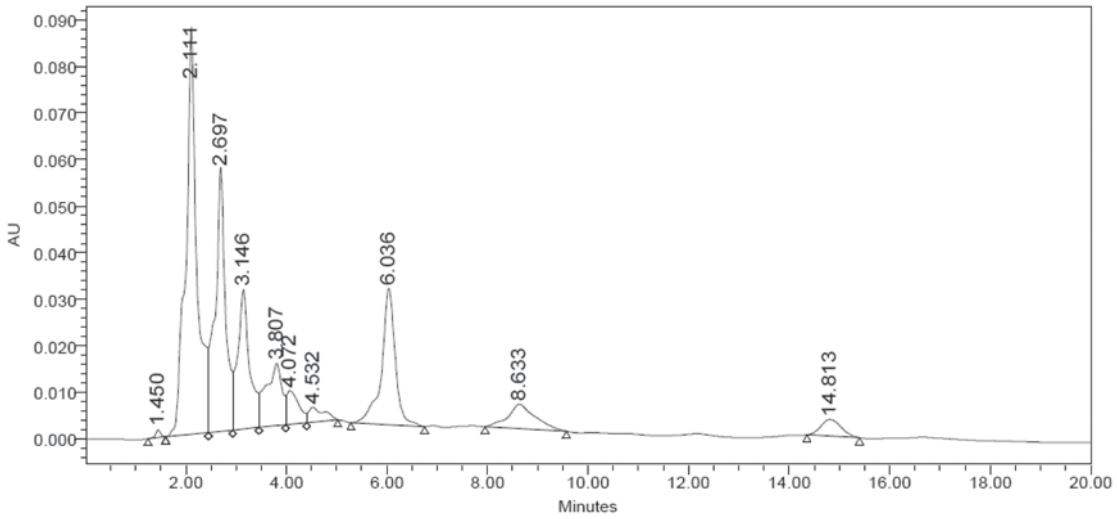

Figure 3. Organic acid produced by *Burkholderia* sp. during phosphate solubilization detected by HPLC analysis.

In dual culture assays, *Burkholderia* sp. recorded 48% growth inhibition against *Fusarium oxysporum* and 50% inhibition against *Phytophthora* sp. In the qualitative estimation assays, the bacterium produced indole-3-acetic acid (IAA), siderophore, hydrogen cyanide, protease, and exopolysachharides (EPS) contributing to systemic resistance. Production of antifugal compounds and compounds inducing systemic resistance were also detected in the P solubilising *P. putida* by Pandey et al. (2008). Overall, use of *Burkholderia* sp. as a bioinoculant will increase the available P in soil and would help reduce the chemical P requirements for crop plants, and would also reduce environmental pollution through chemical fertilizer application.

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