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Isolation and Characterization of Phosphate solubilising *Burkholderia* spp from the crops rhizosphere

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ABSTRACT

It is an important trait in plant growth-promoting bacteria the ability to solubilize and mineralize insoluble inorganic phosphate compounds, making the element available for plants. High populations of phosphate solubilizing bacteria (PSB) will increases the P uptake by plants and reduce the application of organic fertilizers. In this study, a total of 14 PSB isolates from rhizosphere of maize, cotton, ragi, rice, turmeric, sugarcane, cowpea and green gram were tested for the mineral phosphate solubilizing (MPS) activities in Hydroxy Apatite (HAP) broth by analyzing the soluble-P content after 72 h of incubation at 30 ^{0}C. The 'P' solubilising ability of SGN 1 was found to be 35.56 mg/100ml isolated from sugarcane rhizosphere soil. SGN 1 which was showing higher phosphate solubilizing ability in HAP broth was selected for molecular characterization with amplifying and sequencing of 1.3 kb 16S rRNA gene. Based on the BLASTn homology it was found that PSB sugarcane isolate SGN1 having 95% identity with showed 99% homology with *Burkholderia thailandensis.*

Key words: Isolation, *Burkholderia thailandensis*, Phosphate solubilizing, Rhizosphere

Phosphorus has a vital role in plant growth and is found in every living plant cell (Ezawa *et al.*, 2002). It is involved in several key functions of plant, including energy transfer, photosynthesis, transformation of sugars and starches, grain development and nutrient movement within the plant (Arnon, 1956). It provides vigorous start to plant and strengthen straw and decreases lodging tendency. The overall P use efficiency following phosphate fertilizer application is low because of the formation of insoluble complexes (Vassilev and Vassileva, 2003). Plants are unable to use the mineral phosphate directly so there is a need of conversion of mineral phosphate into soluble form. This phenomenon is naturally associated with some soil borne microorganisms having the ability of solubilizing mineral phosphate (Kapoor *et al*., 1996).

Phosphorous is the most limiting nutrient in tropical soil, only 0.1% of the total P present is available to the plants because of its chemical bonding and low solubility (Tilak *et al*., 2005). However, many soil microorganisms have the ability to solubilize and mineralize insoluble inorganic phosphate compounds, such as tri calcium phosphate, di calcium phosphate, hydroxyapatite and rock phosphate, making the element available for plants. Phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+} depending on the particular properties of a soil. In these forms, P is highly insoluble and unavailable to plants. As the results, the amount available to plants is usually a small proportion of this total. Thus, the release of insoluble and fixed forms of phosphorus is an important aspect of increasing soil phosphorus availability. It is generally accepted that the major mechanism of mineral phosphate solubilisation is the action of organic acids synthesized by soil microorganisms. These reactions take place in the rhizosphere and because phosphate-solubilising microorganisms render more phosphates into soluble form than is required for their growth and metabolism, the surplus gets absorbed by plants. (Vassilev *et al*., 2006).

The PSB solubilize the fixed soil P and applied phosphates resulting in higher crop yields (Gull *et al.*, 2004). In view of environmental concerns and current developments in sustainability, research

efforts are concentrated on elaboration of techniques that involve the use of less expensive, though less bio-available sources of plant nutrients such as rock phosphate and by application of PSB the agronomic effectiveness can be enhanced (Whitelaw, 2000). Direct application of phosphate rock is often ineffective in the short time period of most annual crops (Goenadi *et al.*, 2000). Acid producing microorganisms are able to enhance the solubilization of phosphatic rock (Gyaneshwar *et al.*, 2002). The utilization of phosphate-solubilising microorganisms, account for about 45 % of the total biofertiliser production and use (Kudashev, 1956; Krasilinikov, 1957). Use of PSMs can increase crop yields up to 70 percent (Verma, 1993).

A considerable number of bacterial species are able to exert a beneficial effect upon plant growth. Mostly they are associated with the plant rhizosphere, so they are called as rhizobacteria. This group of bacteria has been termed plant growth promoting rhizobacteria (PGPR), and among them are strains from genera such as *Alcaligenes, Acinetobacter, Arthrobacter, Azospirillum, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Paenibacillus, Pseudomonas, Rhizobium* and *Serratia.* Over the past two decades, research on *Burkholderia* species has been steadily expanding. Members of the genus *Burkholderia* are very abundant, occupying diverse ecological niches (Estrada-de los Santos *et al*., 2001), including soil (Janssen, 2006). It can be free-living in the rhizosphere as well as epiphytic and endophytic (Compant *et al*., 2008). Other than P solubilization *Burkholderia* sp. also having many other beneficial plant growth promoting activities like controlling the plant pathogens (biocontrol), production of IAA and siderospores for promoting the crop growth (Pandey *et al.*, 2008). These findings have stimulated a growing interest in using *Burkholderia* sp. isolates in agriculture.

MATERIALS AND METHODS

Isolation and characterization of the bacterial isolates

Soil samples were collected from the rhizospheres of maize, cotton, ragi, rice, turmeric, sugarcane, cowpea and green gram plants from various field location of Tamil Nadu Agricultural University, Coimbatore. A total of 20 composite soil samples were used to isolate phosphate solubilizing *Burkhoderia* sp. by plating on *Burkhoderia* selective BAZ medium with ammonium sulphate as N source and HAP medium. Pour plate method was followed to isolate *Burkholderia* on BAZ agar medium at pH 7.0. The soil samples were serially diluted up to 10^{-4} dilution and one ml of 10^{-4} dilution was transferred to sterile Petriplates and BAZ agar medium was poured and mixed throughly. The plates were incubated under aerobic condition at room temperature for 96 hours. Colonies were observed on the surface of agar plates, they were sub-cultured in again slants for further studies.

Cultural characteristics of the isolates were studied by streaking it on BAZ agar medium in Petriplates. Colony characters like size, margin, elevation and optical characteristics were observed after 48 hours (Fig 1). The morphological characterizations of isolates were made after staining the cultures by gram staining. After staining slides were observed under microscope to visualize the cell morphology.

Screening of phosphate solubilising bacteria *Burkhoderia sp.*

Colonies were taken from the BAZ agar medium and streaked on the Hydroxy apatite (HAP) medium. The pH of the medium was 7.0. The plates were incubated at 30 ºC and maintained for 3 days in HAP medium to observe the halo produced by the isolates in the medium. The isolates with 'P' solubilising ability formed halo zones after 48 to 72 hours incubation. Those isolates which showed phosphate solubilisation potential in HAP medium was screened for 'P' solubilising ability in qualitatively (Fig 2).

Estimation of soluble phosphate in culture filtrate (quantitative assay)

The quantum of soluble phosphate in the culture filtrates of all isolates was estimated. Cultures were inoculated to 100 ml of HAP liquid broth at pH 7 taken in Erlenmeyar flasks. The flasks were aerated by keeping in shaker cum incubator at 180 rpm and 30 ^{0}C temperature and allowed for 5 days. Then this culture was centrifuged at 10,000 rpm for 10 min and the clear supernatant was analysed for the presence of soluble 'P' following method described by Olsen *et al.* (1954).

Molecular characterization of the isolate:

Genomic DNA isolation was carried out from the isolates by following the protocol of

Sambrook and Russell (2001). The total genomic DNA isolated from *Burkholderia* isolates was amplified by PCR, which was performed using the Eppendorf Master Cycler, Gradient (Eppendorf, Germany**).** PCR amplification was carried out using 16S rRNA universal primer (Marchesi *et al*., 1998). The details of primers used to amplify 16S ribosomal RNA (16S rRNA) gene are given in Table 1.

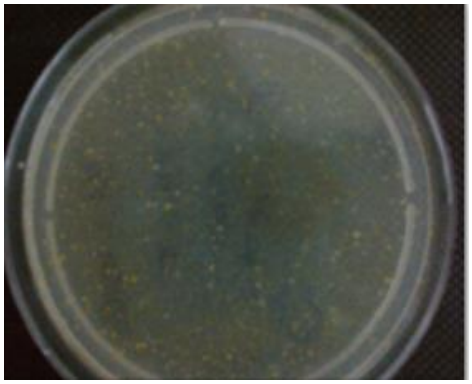

Fig 1. Isolation of *Burkholderia* isolates on BAZ agar medium

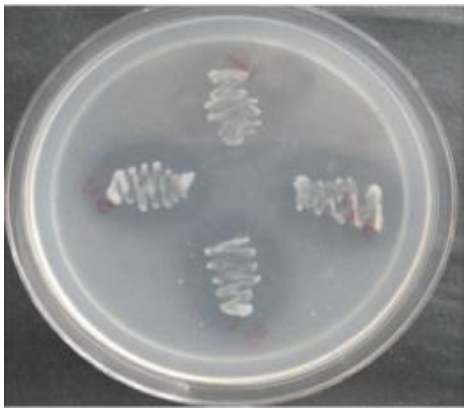

Fig 2. Screening of P solubilising *Burkholderia* isolates on HAP medium

Steps followed in PCR, temperature profile and time duration of each step for amplification of 16S rRNA are given in Table 2. The amplified 16S rRNA gene PCR product of SGN 1 isolate were sent for sequencing to SciGenome Labs Pvt Ltd., Cochin and sequenced through single pass analysis from forward and reverse direction. Sequencing was done by using Automated sequencer.

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Table 1. Details of primers used for amplification of 16S rRNA gene (Marchesi *et al*., 1998)

Target gene	Primer	Primer sequence (5' – 3')
16S rRNA	63f	CAGGCCTAACACATGCAAGTC
	1387r	GGGCGGWGTGTACAAGGC

Table 2. Temperature profile for amplification of 16S rRNA gene by PCR

Sl. No.	Step	Tm (°C)	Time
1.	Initial denature	95	5 min
2.	Denature	94	1 min
3.	Annealing	52	45 sec
4.	Extension	72	1 min
5.	Step 2 to 4	30 cycles	
6.	Final Extension	72	10 min
7.	Hold	4	For ever

Table 3. List of Burkholderia isolates obtained from various fields of TNAU

Sl.No	Crops	Isolate name
1	Maize	MZE 1,2
2	Cotton	CTN1, 2,
3	Ragi	RGI 1
4	Rice	RCE 1,2
5	Turmeric	TMC1
6	Sugarcane	SGN 1,2,3,
7	Cowpea	CPA 1
8	Greengram	GGM 1,2

RESULTS AND DISCUSSION

Studies on phosphate solubilising microbes are gaining importance due to phosphate immobilisation in soil. In this study an attempt was made to isolate efficient 'P' solubilising microorganisms from rhizosphere of different plants and characterise them.

Isolation, cultural and morphological characterization *Burkholderia sp.*

Among the 25 soil samples used to isolate *Burkholderia*, only 19 samples exhibited colonies on BAZ agar medium after 4 days of incubation. Colonies were, Small (1 to 2 mm), circular, pale yellowish brown and translucent at the margins. All these isolates were found to be rod shaped and Gram negative which showed the possibility of these isolates being *Burkholderia sp.* Similar morphology

of *Burkholderia* and Gram reaction were reported by Linu *et al.* (2009).

Screening of *Burkholderia sp.* for 'P' solubilization

Isolates having phosphate solubilising ability produced halo zone due to solubilisation of insoluble mineral phosphate on HAP after 3 days incubation. Out of 19 *Burkholderia* isolates only 14 produced halo zone on HAP (3 days) (Table 3). The quantitative assay was done to find out the amount of soluble phosphate present in the 5 day old culture filtrate from HAP medium for all the 14 isolates. In the present investigation the 'P' solubilizing ability of *Burkholderia sp*. was varied between 35.6 mg/100 ml to 19.8 mg/100ml. Isolates SGN 1 recorded the highest P solubilisation of (35.6 mg/100ml) and next to this was GGM 2 (28.0 mg/100ml), whereas SGN 2 isolate recorded the lowest phosphate solubilisation (19.8 mg/100ml) in HAP broth (Table 4).

It is generally accepted that the mechanism of mineral phosphate solubilisation by PSB strains is associated with the release of low molecular weight organic acids (Goldstein, 1995; Halder *et al*., 1990) which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Kpomblekou and Tabatabai, 1994). However, P-solubilisation is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the culture (Reyes *et al*., 1999). So higher concentration of sugar may increase the low molecular weight organic acids production so that P solubilisation also increased. Son *et al*. (2006) also showed that phosphate solubilisation increased with increasing amounts of glucose up to 3% (w/v) but decreased thereafter.

However, Linu *et al.* (2009) reported up to 68.95mg/ 100ml of soluble phosphorus in the National Botanical Research Institute's Phosphate (NBRIP) medium. This difference might be due to the medium employed by them. 'P' solubilizing ability was found to be high in isolate from bhendi rhizosphere in the present investigation, whereas Linu *et al.* (2009) reported high 'P' solubilizing ability in cowpea rhizosphere. This indicates efficacy of 'P' solubilisation by *Burkhloderia* varies from crop to crop. The presence of *Burkholderia sp.* in the rhizosphere of maize (Paulina *et al*., 2002), *Mimosa* (Pandey *et al*., 2005) and sugarcane (Danice *et al*., 2009) was already reported. In the present study also *Burkholderia* sp. from the rhizosphere of above crops were collected and these isolates showed less 'P' solubilising ability.

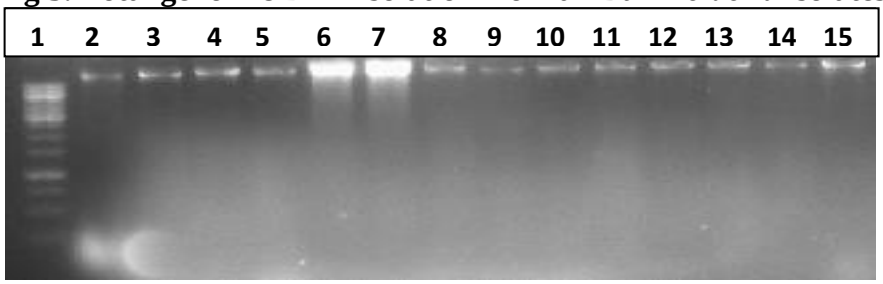
Molecular characterisation of isolates

Total genomic DNA was isolated from the selected isolates produced intact band on 0.8 per cent agarose gel confirmed that isolated DNA was pure and free from protein and RNA contamination (Fig 3). The quantity of DNA was assessed by Spectrophotometry using Nanodrop. Concentration of the DNA was varied from 280 ng/µl to 3200 ng/µl. The OD_{260}/OD_{280} ranged between 1.83 and 2.03 indicating that there was no protein and RNA contamination. When the genomic DNA of *Burkholderia* isolates were amplified by PCR using 16S rRNA primers, they yielded 1.3 kb band which was notified by running the PCR product on 1.2% agarase gel (Fig 4). Nucleotide sequence of the 16S rRNA region of the genome of the isolate SGN 1 was sequenced by using automated sequencer. The sequences were blasted against NCBI database. It was found that SGN 1 isolate (not yet submitted to NCBI) showed 99% homology with *Burkholderia thailandensis.*

Table 4. Estimation of soluble phosphate in culture filtrate of the PSB isolates

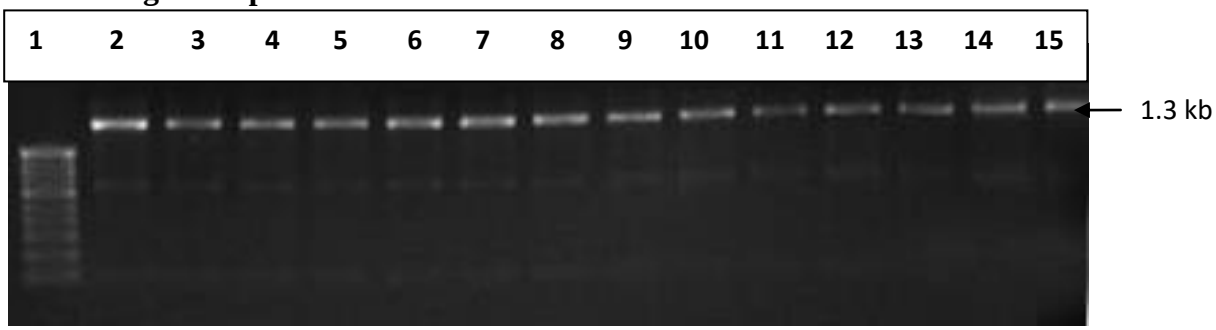
Sl.No.	Isolates	P mg/100ml HAP broth	Sl.No.	Isolates	P mg /100ml HAP broth
1	MZE 1	25.4	8	TMC 1	21.3
2	MZE 2	22.3	9	SGN 1	35.56
3	CTN 1	20.6	10	SGN 2	19.8
4	CTN 2	27.0	11	SGN 3	26.4
5	RGI 1	25.7	12	CPA 1	22.7
6	RCE 1	26.6	13	GGM 1	25.9
7	RCE 2	26.8	14	GGM 2	28.0

Fig 3. Total genomic DNA isolation from all *Burkholderia* isolates



1-1kb adder, 2-MZE 1, 3-MZE 2, 4- CTN 1, 5-CTN 2, 6- RGI 1, 7-RCE 1, 8-RCE 2
9-TMC 1, 10-SGN 1, 11- SGN 2, 12- SGN 3, 13- CPA 1, 14-GGM 1, 15-GGM 2

Fig 4. Amplification of 16S rRNA from all *Burkholderia* isolates



1-1kb adder, 2-MZE 1, 3-MZE 2, 4- CTN 1, 5-CTN 2, 6- RGI 1, 7-RCE 1, 8-RCE 2
9-TMC 1, 10-SGN 1, 11- SGN 2, 12- SGN 3, 13- CPA 1, 14-GGM 1, 15-GGM 2

CONCLUSION

The presence and distribution of *Burkholderia* was found in the rhizospheres of maize, cotton, ragi, rice, turmeric, sugarcane, cowpea and green gram plants. Most of these isolates have phosphate solubilising potential. As *Burkholderia* also have other beneficial effects like N_2 fixation, nodulation, sequestration of iron, ACC deaminase activities and phyto-hormone production, provide resistance to other pathogens, possess symbiotic relationship with beneficial fungi, possess bio control activities, siderophore production, antibiotic production, elicitor of plant defence, induce ISR and other mechanisms to tolerate abiotic stress in plants. This can be exploited as a bioinoculant that have multiple benefits. 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 as enhance the crop growth and its production in various ways.

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