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Research Article

IN VITRO STUDIES ON CHARACTERIZATION AND INVESTIGATION OF *BURKHOLDERIA* SP. FOR POTENTIAL SOLUBILIZATION OF MINERAL NUTRIENTS

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Abstract- The present investigation aimed to develop a potential bioinoculant with *Burkholderia* isolates. Hence isolates were taken from the root, stem and leaf samples of four different crops *viz*., Rice, Maize, Sugarcane and Black gram. The isolates which have provided maximum growth in the N-free BAz medium were further characterized morphologically and biochemically, when compared with the standard strains the organisms were identified as *Burkholderia* isolates (RB_1, MB_2, SB_3 and BB_4). They were produced yellow colour colonies with round and convex in shape, motile in nature and also produced pale yellow surface pellicle, when grown on N-free BAz semi solid medium. All the isolates were gram negative and showed positive reaction for catalase activity, citrate utilization, lipase activity, methyl red, nitrate reduction and gelatin hydrolysis as that of reference strains. The different nutrient sources were used by the *Burkholderia* isolates, comparatively better growth in the carbon sources cellulose and glucose as well as nitrogen sources tryptamine and citrullin. The growth of reference strain *B. vietnamiensis* and the isolates MB_2 and SB_3 showed better growth at pH 5 and 6, whereas the isolates RB_1 and BB_4 showed higher growth that pH 7. The isolate BB_4 recorded maximum titrable acidity and soluble phosphorus, which was closely followed by SB_3 and other *Burkholderia* isolates. The isolate BB_4 and *B. tropicalis* were able to solubilize the insoluble compounds *viz*., $Zn_3 (PO_4)_2$, Zn O, Zn CO_3, $Ca_3 (PO_4)_2$, $Mg_3 (PO_4)_2$ and rock phosphate. None of the isolates and reference strains solubilizes magnesium tri silicate, nickel phosphate and iron sulphate.

Keywords- Endophytes, Biochemical activity, Polysaccharide production, Soluble phosphorus, Titrable acidity, Mineral solubilization

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Introduction

Burkholderia, an important endophytic bacterium was originally described as *Pseudomonas cepacia* in 1950s as a causative agent of bacterial rot of onion bulbs [1]. Though originally it has been identified as a clinical pathogen, *Burkholderia* strains have also been studied for their beneficial properties as well. In particular they were widely studied for their biocontrol mechanisms against fungal pathogens. Hence these bacteria are largely exploited for biocontrol, bioremediation and plant growth promotion purposes [2]. Also nitrogen fixation and the ability to degrade xenobiotic compounds including industrial wastes such as PAH and pesticides were also reported [2]. The studies on the beneficial role of *Burkholderia* sp. indicate their versatility in mineral solubilization in soil. It is well known fact that P-solubilization is important for plant growth because P an essential nutritional element for plants, is insoluble in soil due to ions. According to the reports, usually less than 5 percent of total soil phosphate being available to plants [3-4]. With regard to this, a large number of P-solubilizing bacteria (PSB) have been isolated from the rhizosphere of several crops [5-6]. Interestingly *Burkholderia* sp. has been reported to possess phosphorus solubilizing ability, due to PEP produced by the intermolecular rearrangement of phosphonopyruvate by PEP phosphomutase. This would readily enter intermediary metabolism, serving as a carbon and phosphorus source with excess phosphorus being excreted as Pi [7]. *B. cepacia* a well known species of *Burkholderia* possesses enzymes which facilitate cleavage of the C-P bond of phosphonopyruvate, thereby allowing the organism to mineralize L-phosphonoalanine [8]. It was reported a phosphate regulated gene from *B. cepacia* influencing phosphatase activity, which provide the efficiency of phosphate accumulation in *B. cepacia* and other similar plant growth promoting bacteria [9]. *B. cepacia* by induction of stress protein in response to either an acid environment or high internal phosphate levels leads to increased polyphosphate kinase (PPK) activity and hence poly phosphate accumulation [10]. In line with this, Zinc solubilization is reported to be an important property of organisms as they were in insoluble state in the soil [11-12]. Frequently, microorganisms need to solubilize insoluble metal compounds occurring in the natural environment prior to uptake of essential metals and utilization of associated nutrients [13-15]. Zinc solubilisation ability of *Pseudomonas* sp. has been reported various scientists. It was proved that increased populations of both the non-fluorescent and fluorescent *Pseudomonas* in the rhizosphere of Zn deficient plant, mainly due to loss of membrane integrity in the plant system resulting in increased membrane leakage of organic materials, supporting high bacterial population [16]. Further reports suggest that *Burkholderia stabilis* and *B. multivorans* are positive for extracellular protease activity [17] and *B. cepacia* produces at least, one extracellular zinc metalloprotease that may be involved in virulence [18]. In our earlier study occurrence, screening and nitrogen fixing ability of *Burkholderia* isolates were reported from the root, stem and leaf samples of four different crops *viz*., rice, maize, sugarcane and black gram [19]. The possibility of using them as bioinoculants was also initiated. Considering the versatile behaviour of *Burkholderia*, the isolates were characterised and further *in vitro* studies on mineralisation of insoluble compounds has been initiated in the isolated species with aim of developing a novel bioinculant.

Materials and Methods

Reference strains

The study has utilized the reference strains *Burkholderia vietnamiensis* and *Burkholderia tropicalis* which were procured from Prof. P. Vandamme, Gent University, Belgium and Dr. J. Balandreau, Institute of Research for Development, South Africa respectively.

Characterization of the endophytic *Burkholderia* isolates

The *Burkholderia* isolates *viz*., RB1 (Rice *Burkholderia* 1), MB2 (Maize *Burkholderia* 2), SB3 (Sugarcane *Burkholderia* 3) and BB4 (Black gram *Burkholderia* 4) were selected for further studies were subjected to a set of morphological and biochemical tests as detailed below

Morphological test

The following morphological and biochemical tests were carried out to characterize the endophytic *Burkholderia* cultures.

a) **Colony characters**

The colony characters *viz*., colour, shape and margin were observed on BAZ agar medium [20].

b) **Motility test**

Motility of the isolates was tested using motility medium [21]. The culture was stabbed at the centre of the tube and incubated at 28ºC for 24 hrs. Motile strains were observed due to diffuse growth into medium away from stab line.

Biochemical characterization of the *Burkholderia* isolates

The following biochemical test was carried out according to the procedures mentioned

Gram staining

Gram staining of the isolates was carried out as per Hucker's modified method [22]. Thin smears of the culture were made on separate glass slides, air dried, heat fixed and the smear was covered with crystal violet for 30 sec, followed by a wash with distilled water for a few seconds. The smear was again covered with iodine solution for 30 sec and washed with 95 percent ethanol, then by distilled water and dried. Safranin was applied over the smear for 30 sec, again washed with distilled water, blot dried, air-dried and the cellular morphology was observed under microscope.

Kovac's oxidase test

Using a sterile tooth pick, well-isolated colony was picked and thoroughly rubbed into an area of the moist test disc (impregnated) with oxidase reagent [23]. After 30 seconds, inoculated area was observed for colour change. A bluish purple colour indicated a positive reaction.

Nitrate reduction test

Nitrate reduction test was carried out according to [24]. From Nutrient broth a loopful of broth culture was inoculated in to a tube of nitrate broth and incubated for 7 days at room temperature.

After 7 days incubation period, 1ml of α-Naphthylamine reagent and 1 ml of sulphanilic acid reagent were added to the culture. The appearance of red colour within 30 seconds indicated the presence of nitrate.

Hydrogen sulfide production

Sulfide indole motility (SIM) agar tubes were stab inoculated with the *Burkholderia* isolates and incubated at 35ºC for 48 hrs. Black colouration along the line of stab inoculation indicates H_2S production [23].

Catalase test

Cultures grown on BAz slants for 24 to 48 hours were flooded with 0.5 ml of 3 percent hydrogen peroxide. Rapid effervescence shows positive result for catalase activity [25].

Gelatin liquefaction test

The isolates inoculated in nutrient-gelatin tubes were incubated for 48 hrs in a refrigerator. Deep gelatin inoculated tubes that remain liquefied produce gelatinase and show positive test for gelatin hydrolysis and those tubes that remain solid demonstrate negative reaction for gelatin hydrolysis [26].

Starch hydrolysis test

The isolates to be tested for starch hydrolysis were streaked on starch agar medium and incubated for 48 hrs. The flooded starch agar plates with Gram's iodine were observed for a clear zone underneath and around the growth as an indicator for starch hydrolysis [23].

Urease test

Urease test was performed on 5ml urea broth in test tubes containing phenol red (pH 6.8) as the pH indicator. The urea broth tubes inoculated with isolates were incubated for 24 hrs. The development of red colour in the broth indicated positive reaction for the test [27].

Phenylalanine deaminase test

Tubes containing phenylalanine deaminase broth were inoculated with isolates and incubated at 37ºC for 2 days. They were observed for gas production and pH changes.

Utilization of Citrate test

Citrate test was performed to find out the ability of the isolates to utilize or ferment citrate as the sole carbon source. The test was carried out on Simmon's citrate agar plates. Change in colour of the medium from apple green to blue was positive for the test [28].

Indole production test

The indole test was performed by inoculating the bacterial isolates in to BAz broth. The indole production during the growth was detected by adding Kovac's reagent (P-dimethyl amino benzaldehyde), which produced cherry red colour for positive reaction [29].

Methyl Red-Voges Proskauer test (MR-VP test)

Methyl Red-Voges Proskauer tests were used to differentiate acid producers from those producing a neutral product, acetoin. The isolates were inoculated in 5 ml MRVP tubes and incubated at 35ºC for 48 hours. Methyl red positive tubes were observed by the change of the colour of the media from yellow to red. Voges-Proskauer test were recorded positive by the development of red colour due to the addition of Baritt's reagent-I and Baritt's reagent-II [30].

Over oxidation property

The medium described by [31] was prepared in 50 ml lots in 250 ml flasks. To this medium, ethanol was added at 1 percent concentration before pouring the medium into the petriplates. While pouring the media, care was taken to transfer the entire content of the medium completely to the petriplates. After solidification of the medium, *Burkholderia* cultures were streaked in the plates and incubated at 29ºC. After 5 days, the acetic acid bacterial colonies were identified based on the formation of clear zone.

Lipase activity

Lipase activity was performed on egg-yolk agar. *Burkholderia* isolates were inoculated on egg-yolk agar and incubated at 37ºC for 48 hrs. The development of opalescent precipitates indicated positive reaction of the lipase activity [32].

Polysaccharide production

Polysaccharide production was performed on BAz agar medium. *Burkholderia* isolates were streaked on BAz agar and incubated at 37ºC for 48 hrs. The polysaccharide production was observed around the colonies.

Growth behavior of *Burkholderia* isolates

Determination of bacterial growth by turbidity measurements

The log phase cultures of isolates were transferred to BAz broth in flasks.

The inoculated culture flasks were kept in shaker at 120 rpm and the OD value was taken at 620 nm in a colorimeter at 2 hours interval. The graph was plotted against absorbance and time [32].

Determination of growth on different carbon sources

Exactly 100 ml of BAz broth was prepared in 250 ml side arm flask with different carbon sources *viz*., glucose, galactose, glycerol, mannitol, sorbitol, lactose, sucrose, cellulose, xylose and malic acid. It was sterilized in the autoclave at 121ºC for 20 minutes. The log phase culture was inoculated into BAz broth containing different carbon sources. After 48 hrs, the growth was read at 620 nm in a colorimeter against control maintained without carbon source.

Determination of growth on different pH

Exactly 100 ml of nutrient broth was prepared in a 250 ml side arm flask adjusted with different pH (3, 4, 5, 6, 7, 8, 9 and 10). It was sterilized in the autoclave at 121ºC for 20 minutes. The log phase culture was inoculated into nutrient broth having pH 3, 4, 5, 6, 7, 8, 9 and 10. After 48 hrs, the growth in the culture broth was read at 620 nm.

Determination of growth on different nitrogen sources

Exactly 100 ml of BAz broth was prepared in a 250 ml side arm flask with different nitrogen source viz., tryptamine, citrulline, ammonium sulphate, ammonium nitrate, yeast extract and peptone, sterilized in the autoclave at 121ºC for 20 minutes. The log phase culture was inoculated into each flask and appropriate control was maintained without nitrogen source. After 48 hrs, the growth was measured in a colorimeter at 620 nm.

Determination of soluble phosphorus and titrable acidity

The growth medium after 7 days of incubation was centrifuged at 10,000 rpm for 10 minutes and the clear supernatant was analyzed for soluble phosphorus content following the method described by [33]. A quantity of 1 ml of the culture filtrate was pipetted out into 25 ml volumetric flasks and diluted to 20 ml with distilled water. Four ml of the reagent B were added to the flask. The intensity of blue colour developed was read at 660 nm in Systronics photoelectric calorimeter using appropriate reagent blank. The standard curve was prepared with potassium di hydrogen orthophosphate (KH_2PO_4) and the quantity of 'P' solubilized was determined by referring the standard graph.

Estimation of titrable acidity in the culture filtrates

The culture medium was centrifuged at 10,000 rpm for 10 minutes to remove the cells and traces of tri calcium phosphate. Five ml of the supernatant was pipetted out into 0.01 N NaOH using phenopthalin as indicator. The titrable acidity was expressed as ml of 0.01 N NaOH consumed per 5 ml of culture filtrate.

Determination of zinc and other insoluble compounds solubilization using Tris-minimal salt medium

Zinc and other mineral solubilization by the *Burkholderia* isolates were determined as per the method described by [34]. The medium used in the study was Tris-Minimal Salt medium with 0.43 g l^{-1} of ammonium sulphate as N source. The medium was prepared by incorporating insoluble zinc sources and other insoluble compounds *viz*., zinc oxide, zinc carbonate and zinc phosphate, magnesium tri silicate, magnesium phosphate, tri calcium phosphate, iron sulphate and nickel phosphate at 0.1 percent. The pH was adjusted to 5.7 after sterilization by using sterilized 0.1 N KOH and HCl, the media was shaken for uniform distribution of the zinc and other mineral insoluble source into the petriplates. Ten µl of *Burkholderia* suspension was placed to agar plates and observed for solubilization zones upto 5 days.

Statistical analysis

The data generated from the experiment were statistically analysed as per the procedure suggested by [35]. Critical differences were worked out at 5% probability level and presented.

Results and Discussion

Group of beneficial rhizobacteria especially *Rhizobium, Azospirillum, Azotobacter etc*. have been used extensively world-wide as bacterial inoculant for higher crop productivity [36]. In recent years there are encouraging reports about the use of dinitrogen fixing bacteria in combination with phosphate solubilizing and plant growth promoting rhizobacteria for meeting the nutritional requirements of crops and supplementing the expensive renewable inorganic fertilizers [37]. Members of the genus *Burkholderia* are versatile organisms that occupy a surprisingly wide range of ecological niches. Considering all these a total of thirteen isolates was obtained in our previous study from roots, stem and leaf *viz*., rice, maize, sugarcane and blackgram. The isolates were morphologically characterized, and the results were presented in [Table-1]. The isolates of RB_1, MB_2, SB_3 and BB_4 which have shown nitrogen fixing ability has been chosen for further studies. For a long time, N_2 fixing ability of the genus *Burkholderia* was recognized only in the species *B. vietnamiensis* [20]. Recently, *B. kururiensis*, which has endophytic association with maize, sorghum and coffee plants also showed the nitrogen fixing ability [38]. The same has been depicted in our study as the four isolates from different crops have shown nitrogen fixing ability by in vitro studies and they were subjected to morphological and biochemical characterization studies.

Morphological characterization of *Burkholderia* isolates

The *Burkholderia* isolates showed different color properties when grown on agar, semisolid and liquid broth of BAz medium. All the isolates exhibited pale yellow surface pellicles on N-free BAz semi solid medium and when grown on N-free BAz agar medium color change from green to blue colour was noticed. The same when grown on BAz broth, they turned it to the yellow colour. Earlier N-fixing isolates when tested in the N-free semi solid BMGM (modified BAz) medium and many N-fixing isolates formed white colonies, while other formed in whitish or yellowish colonies on BAc agar [38]. In our study all the colonies were round and smooth with entire margins varying in diameter from 1 to 2 mm. White colonies were flat or slightly convex and these isolates turned the medium from green to deep blue. While, isolates whitish or yellowish colonies were convex and turned the medium a light blue colour. Though concrete conclusions cannot be derived with this the results proved that the organisms belonged to genus *Burkholderia* and have ability to fix atmospheric nitrogen into the soil. Hence further studies were initiated to biochemically characterized the organisms.

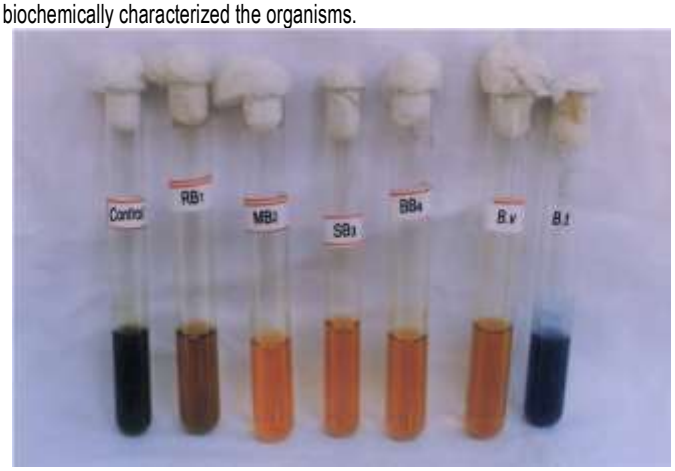

Fig-1 Growth of *Burkholderia* sp. on N-free BAz semi-solid medium

Biochemical characterization of *Burkholderia* isolates

The biochemical characters of selected *Burkholderia* isolates *viz*., RB_1, MB_2, SB_3 and BB_4 are presented in [Table-2]. In present experiment, the new isolates of *Burkholderia* from different crops were characterized and compared with reference strains *B. vietnamiensis* and *B. tropicalis*. The isolates resembled the type strain *Burkholderia* vietnamiensis, when it was streaked on agar plates. It produced yellow colour colonies, round, convex and polysaccharide production except MB_2 and SB_3. These isolates were confirmed by their gram negative [Fig-2] and motility when it was observed under microscope. When compared with previous reports *B. cepacia* is identified as predominant colonizer of several crops including rice, maize, pea, corn, sunflower and radish [39-42].

The other nitrogen fixing *Burkholderia* species are *B. vietnamiensis* [20], *B. vandii* [43], *B. graminis* [44], *B. multivorans* [45] and *B. thailandensis* [46]. But in our study the isolates majorly belonged to *B. vietnamiensis* and *B. tropicalis*. In addition to these isolates have produced exopolysaccharide (EPS) [Fig-3].which plays a major role in plant bacterial interaction and colonization [47]. Further the growth of isolates on different carbon and nitrogen sources were investigated.

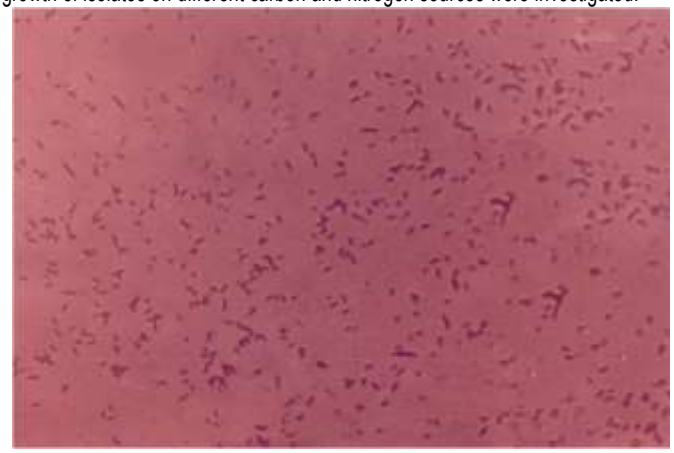

Fig-2 Microscopic view of *Burkholderia* sp.

Fig-3 Polysaccharide production of *Burkholderia* isolates on BAz medium

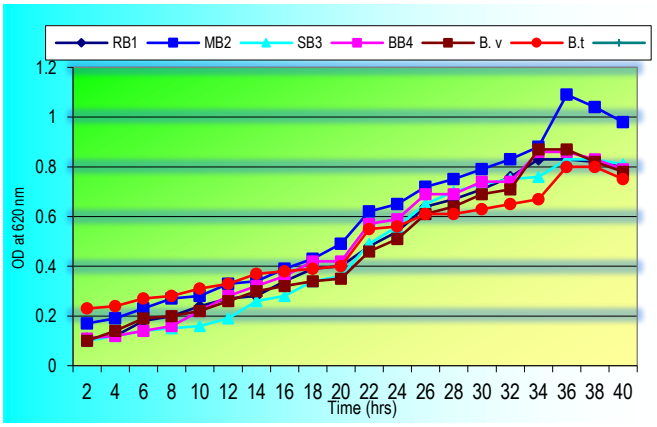

Fig-4 Generation time of *Burkholderia* isolates

Growth behavior of *Burkholderia* isolates

The growth of *Burkholderia* isolates in BAz medium was assessed at two hours intervals and it was seen that the growth of all the isolates started declining after 38 hrs [Fig-4]. The conclusions could not be drawn as the study was not conducted using natural media and the media which have been developed is meant for isolation of Bcc (*Burkholderia cepacia* complex) from clinical samples. Hence it forms an essential part to determine the best carbon and nitrogen sources for growth of the bacteria in lab conditions. On analysis, it has been found that all the *Burkholderia* isolates and reference strains preferred glucose and cellulose as carbon sources than other sugars attempted. However, better growth of RB_1, SB_3 and BB_4 was noticed in mannitol, glycerol and galactose medium [Fig-5]. In short, all *Burkholderia* isolates including standard strain, *B. vietnamiensis* utilized different carbon sources particularly cellulose and glucose effectively than sucrose, malate and lactose. According to the previous reports, *Burkholderia*, a nutritionally versatile bacterium is capable of assimilating a large variety of carbon sources including unusual carbon sources like azelaic acid [48]. Similarly, [43] reported that *Burkholderia* isolates could utilize N-acetylglucosamine, D-aribitol, benzoate, D-glucose, glycerol, inositol, 2-keto gluconate, mannitol, D-mannose, sorbitol and D-xylose as a sole carbon source. In addition, most strains accumulate polyhydroxybutarate as carbon reserve material and are capable of ortho cleavage of protocatechuate [20]. With respect to nitrogen sources, isolates BB_4 and *B. vietnamiensis* exhibited tryptamine and citrullin were suitable nitrogen sources. All the isolates and reference strains utilized tryptamine, citrulline, peptone, and ammonium sulphate and yeast extract. Ammonium nitrates were not utilized by these isolates [Fig-6]. In the same way all the isolates except RB1 showed maximum growth at pH 5 and 6, little growth at pH 9 and low growth at pH 3 [Fig-7]. The results were also reported by [49].

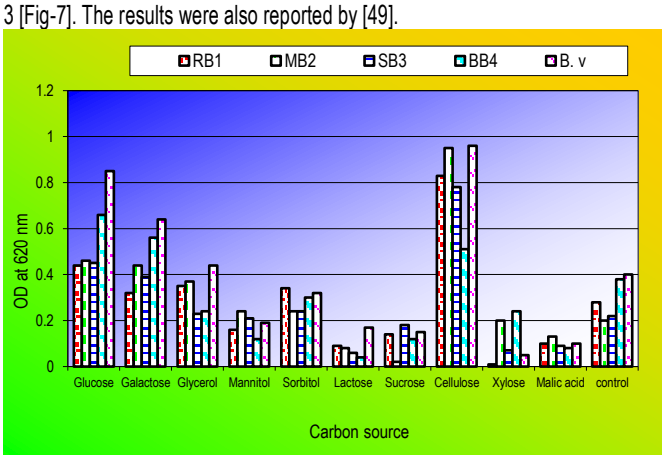

Fig-5 Effect of different carbon sources on the growth of *Burkolderia* isolates

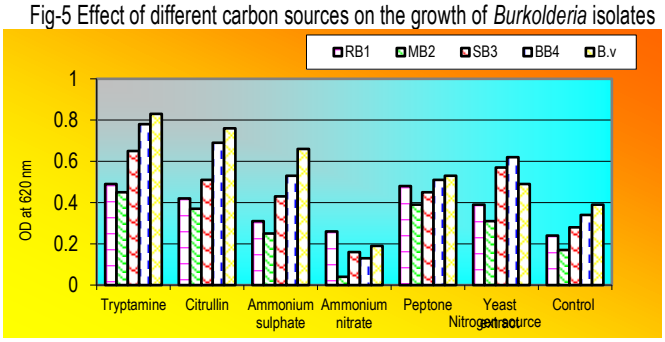

Fig-6 Effect of different nitrogen sources on the growth of *Burkholderia* isolates

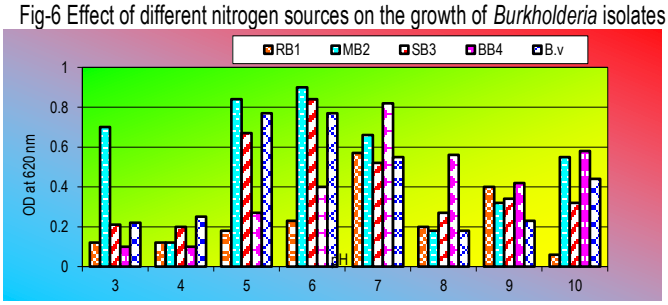

Fig-7 Effect of different pH on the growth of *Burkholderia* isolates

***In vitro* evaluation of beneficial properties *viz.*, Phosphorus solubilisation and Zinc solubilisation of the isolates**

The study analyses the suitability of the isolates as bioinoculant, as their nitrogen fixing property has been studied earlier. Further, phosphorus and zinc solubilisation potential of the isolates were studied. Phosphorus solubilization was observed by all the isolates tested [Table-3]. The highest value was recorded in BB_4 (4.29 µg ml^{-1}) followed by SB_3 (4.25 µg ml^{-1}), MB_2 (4.01 µg ml^{-1}), *Burkholderia tropicalis* (3.69 µg ml^{-1}) and the minimum solubilization of phosphorus was noticed in *B.vietnamiensis* (2.68 µg ml^{-1}).

Table-1 *Morphological characteristics of Burkholderia isolates in BAz medium*

Characters	*Burkholderia* isolates				
	RB_1	MB_2	SB_3	BB_4	B. v*
Colony colour	Yellow	Yellow	Yellow	Yellow	Yellow
Colony shape	Round	Round	Round	Round	Round
Colony margin	Smooth and convex	Convex	Smooth and convex	Convex	Convex
Growth in N-free semi solid medium	Yellow pellicle on surface	Pale yellow coloured pellicle on sub surface	Yellow pellicle on surface	Pale yellow coloured pellicle on sub surface	Pale yellow coloured pellicle on sub surface
Motility	Present	Present	Present	Present	Present

(* - *Burkholderia vietnamiensis* - Reference strain)

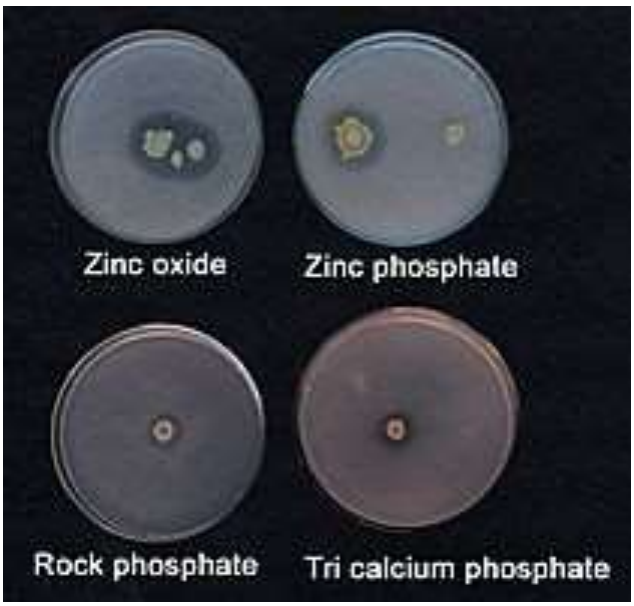

Fig-8 Zinc solubilization of *Burkholderia* sp. on Tris-HCl minimal salt medium

Table-2 *Biochemical characterization of Burkholderia isolates*

SN	Biochemical tests	*Burkholderia* isolates					
		RB_1	MB_2	SB_3	BB_4	*B. v**	*B. t**
1	Gram reaction	-ve	-ve	-ve	-ve	-ve	-ve
2	Oxidase test	-	+	-	+	+	+
3	Nitrate reductase test	+	-	+	+	+	+
4	Hydrogen sulphide production	-	-	-	+	-	-
5	Catalase activity	+	+	+	+	+	+
6	Gelatin hydrolysis	+	+	-	+	+	+
7	Starch hydrolysis	+	-	+	-	-	+
8	Urease test	+	-	-	-	-	-
9	Phenylalanine deaminase test	+	+	-	-	-	+
10	Simmon's citrate test	+	+	+	+	+	+
11	Indole production test	+	-	+	-	-	+
12	Methyl Red test	+	+	+	+	+	-
13	Voges-proskauer test	+	-	+	+	-	-
14	Over oxidation test	+	-	+	+	-	-
15	Lipase activity	+	+	+	+	+	+
16	Polysaccharide production	+	-	-	+	+	+

(* - *Burkholderia vietnamiensis* and *Burkholderia tropicalis* - Reference strains)

Table-3 *Determination of soluble phosphorus and titrable acidity of the Burkholderia isolates in BAz medium*

Burkholderia isolates	Soluble Phosphorus (µg ml^{-1})	Titrable acidity of the culture supernatant (ml of 0.1 N NaOH consumed)
RB_1	3.69	9.7
MB_2	4.01	9.2
SB_3	4.25	10.2
BB_4	4.29	10.4
B. vietnamiensis*	2.68	10.0
*B. tropicalis**	3.69	7.8
SEd	0.07	
CD (5%)	0.16	

(* - *Burkholderia vietnamiensis* and *Burkholderia tropicalis* - Reference strains)

Table-4 *Solubilization of Zinc and some other insoluble compounds by Burkholderia isolates grown in minimal salt medium*

Insoluble compounds	Solubilization *Burkholderia* isolates					
	RB_1	MB_2	SB_3	BB_4	*B.v**	*B.t**
$Zn_3 (PO_4)_2$	+	-	+	+	-	+
Zn O	-	-	+	+	-	+
$Zn CO_3$	+	-	-	+	-	+
$Ca_3 (PO_4)_2$	-	-	-	+	-	+
$Mg_3 (PO_4)_2$	-	+	-	+	+	+
Rock phosphate	+	-	-	+	-	+
Magnesium tri silicate	-	-	-	-	-	-
$Ni_3 (PO_4)_2. 7H_2O$	-	-	-	-	-	-
$Fe SO_4. 7H_2O$	-	-	-	-	-	-

(* - *Burkholderia vietnamiensis* and *Burkholderia tropicalis* - Reference strains)
(+ = Solubilization / positive), (- = Insoluble / negative)

Phosphorus solubilisation is always connected with the acid and enzyme production of the respective bacterial species. Previous studies suggest that the solubilization of organic phosphate is carried out by bacteria with the help of phosphate enzymes, especially acid phosphatase, which play the major role in organic phosphate solubilization in soil [50-51]. In addition, the secretion of organic acids by certain rhizosphere bacteria, cause a localized lowering of the soil pH and a concomitant enhancement of phosphate diffusion [52]. Hence the acid producing ability of the isolates was determined. The amount of acid production in the culture medium due to the bacterial growth was estimated by titrating against standard alkali (0.01 N NaOH) and the results were presented in [Table-3]. The titre value for 5.0 ml of the culture supernatant varied between 7.8 and 10.4 ml of 0.01 N NaOH consumed for various isolates. Among the isolates, BB4 produced higher amount of titrable acidity (10.4 ml) closely followed by SB3 (10.2 ml), B.v (10.0), B.t (9.7 ml) and MB_2 (9.2). The lowest acid production was recorded in RB1 (7.8 ml). The present study also revealed that the *Burkholderia* isolates produce higher amount of soluble phosphorus. This supports the view that acidic pH and product of microbial activity helped in phosphorus solubilization [53]. High P solubilization without much pH change was observed in the case of *Burkholderia* isolate BB_4. Similar observation was reported by [54-55] who observed that high phosphate solubilization without detectable pH change.

Similar to mineral solubilisation, many studies on bacterial interactions with toxic metals have been made in the context of functions in metalloenzymes resistance and transport; particularly the mechanisms employed to obtain metals and associated nutrients from insoluble resources [56]. In this study, *Burkholderia* isolates behaved differently in the experiment conducted to study the solubilization of zinc and some other insoluble compounds [Table-4]. The isolate BB_4 and reference strain *B.tropicalis* solubilized most of the compounds *viz*., $Zn_3 (PO_4)_2$, Zn O, Zn CO_3, $Ca_3 (PO_4)_2$, $Mg_3 (PO_4)_2$ and rock phosphate [Fig-8]. None of the isolate and reference strain solubilized magnesium tri silicate, and Fe SO_4.

These results go on par with the studies of [57] where he stated that Gluconic acid and 2-ketogluconic acids are known to be produced by *Pseudomonas cepacia* during solubilization of rock phosphate. Similar to this in the present investigation, the *Burkholderia* isolates showed the solubilization of zinc and rock phosphate, which was recorded earlier in *Pseudomonas fluoresencens* and *P. aeruginosa* [58], when Zn O and $Zn_3 (PO_4)_2$ were incorporated in the medium. The presence of zinc oxide showed high levels of solubilized zinc, whereas zinc phosphate was not completely solubilized even after 10 days of incubation.

The bacterial solubilization of insoluble zinc oxide and phosphate is mediated by the production of 2-ketogluconic acid, which appears different in several fungi where other carboxylic acids appear to be of greater significance [59]. *Burkholderia cepacia*, *Ralstonia picketti, Klebsiella pneumonia and Pseudomonas aeruginosa* were identified as zinc solubilising bacteria, in which the isolate Ralstonia effectively used as Zn biofortification of rice crop evidenced by [60].

Conclusion

The present investigation brings the versatility of *Burkholderia* isolates in nutrient mobilization especially phosphorus and zinc. The positivity of the isolates can be exploited for plant growth by including them as bioinoculant for which further studies are underway.

Application of research: In this research, we are characterize the Burkholderia isolates of both morphological as well as biochemical experiments and the application of Burkholderia isolates into solubilisation of insoluble minerals for bioaugmentation of nutrients to improve crop growth.

Research Category: Morphological and biochemical characterization

Abbreviations:

RB_1:(Rice *Burkholderia* 1
MB_2: Maize *Burkholderia* 2
SB_3: Sugarcane B*urkholderia* 3
BB_4: Black gram *Burkholderia* 4
BAz: *Burkholderia* Azelaic acid) medium

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