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A Novel Multifunctional *Burkholderia* sp. for Plant Growth Promotion

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***Burkholderia* sp. have been isolated from the rhizosphere soils of different plants, *viz.,* maize, sugarcane, red gram, rice, bhendi (Okra), brinjal, cotton, chili, cowpea *etc.* The isolates were checked for nitrogen fixation, ability to solubilize insoluble phosphate and antagonistic activity against plant pathogenic fungi *Rhizoctonia solani* and *Macrophomina phaseolina* by using different growth media like BMGM, HAP, PDA+NA respectively. The isolates were also checked for nitrogenase activity by using gas chromatography. B1 and R1 isolates showed high nitrogenase activity (63.45 and 62.35 n moles of ethylene/hr./ml respectively). Total genomic DNA was isolated and PCR with 16S rRNA gene specific primers yields amplicons of 1.5 kb size in 4 selected isolates of *Burkholderia* sp. The PCR was carried out with specific primers of nif H gene primer for selected isolates. It showed that selected four isolates were found to have nif H gene with 400 bp. Sequence analysis revealed homology of 16S rRNA amplicons of B1 as *Burkholderia thailandensis* and R1 as *Burkholderia vietnamiensis.* The present study revealed that some of the *Burkholderia* sp. helps in plant growth promoting activities like nitrogen fixation, phosphate solubilization and antagonism to enhance the yield of crop plants that can be exploited as bioinoculant in agriculture.**

Key words: *Burkholderia* sp., rhizosphere, nitrogenase activity, antagonism, bioinoculant.

N_2-fixing ability in bacteria of the genus *Burkholderia* was recognized only in the species *Burkholderia vietnamiensis* (Gillis *et al*. 1995). Recently, the analysis of N_2-fixing bacteria associated with maize and coffee plants grown under field conditions revealed the presence of *B. vietnamiensis*, as well as the richness of novel diazotrophic bacterial species belonging to the genus *Burkholderia* (Estrada-de los Santos *et al*. 2001). Other species of environmental origin were then added to this genus, including *Burkholderia graminis, B. caribiensis, B. kuriensis* (Zhang *et al*. 2000), *B. ubonensis*, *B. caledonica* and *B. fungorum* (Coenye *et al*. 2001), and *B. sacchari* (Bramer *et al*. 2001). Similarly, *B. vietnamiensis* has attracted interest because of its abilities to promote rice plant growth and grain yield.

Phosphorus (P) is one of the major essential macronutrients for biological growth and development. Microorganisms play a central role in the natural phosphorus cycle. They solubilize the bound phosphate present in soil. The phenomena of fixation and precipitation of Phosphate in soil is generally highly dependent on pH and soil type. In addition, studies on the diversity of plant-associated bacteria may contribute to the discovery of new beneficial plant-microbe interactions. Plant root-associated phosphate solubilizing bacteria (PSB) have been considered as one of the possible alternatives for inorganic phosphate fertilizers for promoting plant growth and yield (Thakuria *et al*. 2004). They not

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only provide plants with phosphorus, but also facilitate the growth of plants through fixing atmospheric nitrogen (Sahin *et al*. 2004); accelerating the accessibility of other trace elements (Mittal *et al*. 2008); producing plant hormones such as auxins (Egamberdiyeva 2005), cytokinins, and gibberellins (Gutierrez-Manero *et al*. 2001); releasing siderophores (Wani *et al*. 2007), hydrogen cyanide (Kang *et al*. 2010), enzymes and fungicidal compounds such as chitinase, cellulose, protease (Hamdali *et al*. 2008) which ensure antagonism against phytopathogenic microbes.

Ganyu *et al*. (2009) reported that *Burkholderia* sp. are frequently isolated from the rhizosphere of crops, and are involved in growth promotion of plants and suppression of plant diseases, *Burkholderia contaminans* strain MS14 has a broad range of antifungal activities to plant and human pathogens especially for *Geotrichum candidum*. Therefore, it is worth to believe that production of plant growth promoting substances by microbes may effectively contribute to their effect on the enhancement of the plant performance (Hameeda *et al*. 2006). The main objective of this study is to isolate and characterize *Burkholderia* species from the rhizosphere soils and to assess their ability of nitrogen fixation, solubilize bound phosphate and antagonistic effect on soil borne plant pathogens and molecular screening of the isolates.

MATERIALS AND METHODS

Rhizosphere soils of plants like maize, sugarcane, red gram, rice, bhendi, brinjal, cotton, chilli, cow pea *etc.*, were collected from the fields. Nitrogen free BMGM medium was used for the isolation of *Burkholderia* sp. They were further purified and each isolate was characterized by Gram reaction, morphology, temperature, biochemical tests *etc*. From a total of 30 isolates, the strains which have the ability of Nitrogen fixation and Phosphorus solubilization were selected for further study. List of some selected isolates is given in Table 1.

Characterization of the obtained isolates

The isolates were purified and checked for Gram reaction and characterized biochemically. Exocellular enzymatic tests like gelatin liquefaction, starch hydrolysis, lipid hydrolysis, casein hydrolysis were performed. Also catalase test, MR-VP, indole test, urease test, citrate utilization test, Hydrogen sulphide production test, carbohydrate fermentation test with utilization of sugars like glucose, lactose were performed for all the isolates and the results were recorded (Pandey *et al*. 2005).

Nitrogen fixing ability

The N_2 fixing ability of the isolates were identified by growing the cultures in a N_2 free BMGM broth (Govindarajan *et al*. 2007) at an initial pH of 5.7 by using malate as a carbon source. The sterilized BMGM broth was inoculated with the test isolates and incubated at 37^0C. The alterations in pH were recorded at an interval of every 2 days and results were tabulated. The isolates were further inoculated in nitrogen free BMGM plates and incubated for 3-5 days and colour change of the medium from golden yellow to blue was observed for confirmation of N_2 fixing activity.

Estimation of Nitrogenase activity

The nitrogenase activity was estimated by the acetylene reduction assay based on the reduction of acetylene to ethylene by gas chromatography (Hardy *et al*. 1968). By using sterile syringe, 10 per cent of the air inside was evacuated and replaced with 8.0 ml of pure acetylene in the glass vials containing 60 ml of *Burkholderia* culture. Glass vials were incubated for 24 hrs. and analyzed for ethylene (C_2H_4). Ethylene was analyzed by standard flame ionization detector (FID) gas chromatography standardized with pure ethylene. Peak area of ethylene was measured and recorded. Nitrogenase activity was calculated by using the following formula: where, 1 FID unit = 8.2×10^{-3} n mole, Volume of glass vial = 100ml, hours of incubation after acetylene injection = 24, volume injected = 30µl.

$$\text{Nitrogenase Activity} = \frac{\text{Peak area x FID unit x Volume of glass vial}}{\text{Hours of incubation x Volume of gas injected}}$$

Phosphate solubilization

Burkholderia isolates were inoculated in hydroxyl apatite medium (HAP) (Ayyakkannu and Chandramohan, 1971). The HAP plates were incubated at 37^oC for 2-3 days for assessing the 'P' solubilizing ability of the isolates.

Bio control activity

The antagonistic activity of the isolates

was determined by dual culture method (Rangeswaran and Prasad, 2000). The *Burkholderia* isolates were tested for their ability to inhibit plant pathogenic fungi like *Rhizoctonia solani* and *Macrophomina phaseolina*.

Genomic DNA isolation and Molecular characterization of isolates

Genomic DNA isolation was carried out from the isolates by using the phenol/chloroform/ Isoamyl alcohol method (Lazo *et al*. 1987). The total genomic DNA isolated from *Burkholderia* isolates was amplified by using universal 16S rRNA primer (Table 2).

Amplification of nif H gene primer

The total genomic DNA isolated from *Burkholderia* isolates amplified by using nif H primer. The details of primer used to amplify nif H gene was given in Table 3.

Table 1. List of *Burkholderia* isolates

Sr. No.	Crops	Isolate name
1.	Bhendi	B1
2.	Rice	R1
3.	Maize	M1
4.	Maize	M2
5.	Sugarcane	S1
6.	Sugarcane	S2
7.	Red gram	T1
8.	Red gram	T2
9.	Red gram	T3
10.	Brinjal	BR1
11.	Cow pea	CP1
12.	Chilli	C1
13.	Chilli	C2
14.	Cotton	CO1
15.	Cotton	CO2

Sequencing Analysis

The amplified 16S rRNA gene PCR product of 2 isolates (B1 and R1) 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.

RESULTS AND DISCUSSION

Isolation and Characterization of the *Burkholderia* isolates

Burkholderia isolates were characterized morphologically and results were given in Table 4. The cultures were characterized by Gram staining (Fig. 1) salt, pH and temperature tolerance tests and biochemical tests like Starch hydrolysis, Catalase test, Lipid hydrolysis, Citrate utilization Casein hydrolysis, Gelatin liquefaction, Urease test, Hydrogen sulphide production test, *etc*. were carried out. The obtained results were showed in Table 5. Isolation of *Burkholderia* from root and rhizosphere soil of different plant and have been reported by many authors during their investigation. *Burkholderia* was isolated from rhizosphere soil of wheat and maize using a medium containing azelaic acid and tryptamine as sole carbon and nitrogen sources respectively (Viallard *et al*.1998), sugarcane plantation in Brazil (Bramer *et al*. 2001), sugarcane, maize and teosinte plants in Brazil, Mexico and South Africa (Reis *et al*. 2004), *Mimosa* sp. (Sheu *et al*. 2013), root nodules of *Mimosa caesalpiniifolia* (Chen *et al*. 2008), rhizosphere of maize and from surface-sterilized leaves of sugar cane cultivated in Rio de Janeiro, Brazil (Perin *et al*. 2006), from surface-sterilized roots and stems of different sugarcane varieties in the Tamil Nadu region of India (Govindarajan *et al*.

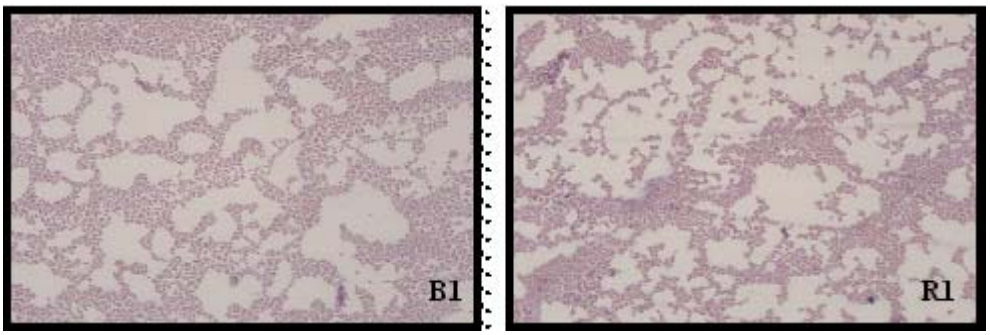


Fig. 1. Microscopic observation of *Burkholderia* isolates (100X)

2007). Morphological characteristics of *Burkholderia* isolates were studied. The isolate was characterized based upon its morphological, colony characteristics and Gram reaction. Colonies were mucoid, slimy, and translucent in nature. The isolate was Gram negative, rod shaped and non-motile. Govindarajan *et al*. (2007) also observed that the *Burkholderia* grown on BMGM medium with similar characters.

Salt tolerance test

The isolates like M1, M2, S1 and S2 can tolerate low salt concentration (0.01%-0.05%). All the 10 isolates grow luxuriantly at a salt concentration of 0.2%-0.8% and optimum being 0.3%. The isolates like R1, B1, CO1 and CO_2 can tolerate high salt content *viz*., 1%, 2% and 3%.

Temperature & pH tolerance test

All the 10 isolates can tolerate a wide range of temperature like 20^0C-45^0C. However temperature tolerance highly depends on the salt concentration. Also they grow luxuriantly in a wide pH range of 5-9.

Nitrogen fixation by *Burkholderia*

The N_2 fixing ability of the isolates was identified by growing the cultures in a N_2 free BMGM medium (Fig. 2) and BMGM broth at an initial pH of 5.7 by using malate as a carbon source (Fig. 3). The sterilized BMGM broth was inoculated

Table 2. Details of primers used for amplification of 16S rRNA gene

Target gene	Primer	Primer sequence	Reference
16S rRNA	63f 1387r	5'-CAGGCCTAACACATGCAAGTC-3' 5'-GGGCGGWGTGTACAAGGC-3'	(Marchesi *et al*. 1998)

Table 3. Details of primer used for amplification of nif H gene

Target gene	Primer	Primer sequence	Reference
Nif H2	Nif H-2f Nif H-2r	5'-CGCCGGCGCAGTCTTTGCCG-3' 5'-CACTCGTTGGAGCTGGTCGG-3'	(Frankae *et al*. 1998).

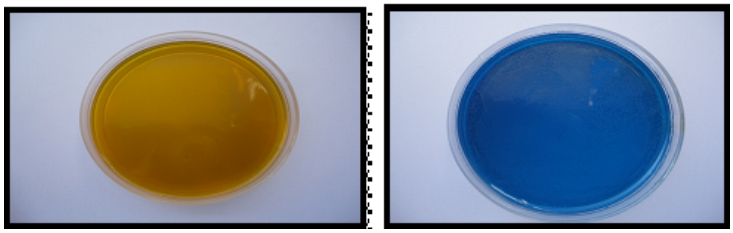


Fig. 2. Change of colour due to pH increase by B1 isolate inoculated in N free BMGM medium

Fig. 3. Nitrogen fixation by *Burkholderia* on N free BMGM broth

with the test isolates and incubated at 37°C. The alterations in pH were recorded at an interval of every 3 days and results were tabulated (Table 6). The isolates were further inoculated in nitrogen free BMGM plates and incubated for 3-5 days and colour change of the medium was observed for confirmation of N_2 fixing activity. In this test four efficient nitrogen fixing *Burkholderia* B1, R1, M2, and S2 were able to change pH of BMGM broth from 5.7 to 9.01, 9.05, 8.9 and 9.01 respectively. Nitrogen fixation by *Burkholderia* isolated from root and rhizosphere soil of different plant were earlier in endophytic sugarcane diazotroph by Govindarajan *et al*. (2007) and Estrada-de los Santos *et al*. (2001) reported that genus *Burkholderia* comprises 19 species, including *Burkholderia vietnamiensis*, which is the only known N_2-fixing species of this bacterial genus and in their investigation most of the N_2-fixing isolates were recovered from the environment of field-grown maize and coffee plants. These reports found to be similar with our findings.

Table 4. Morphological characteristics of *Burkholderia* isolates on BMGM medium

Character	Result
Colony shape	Circular
Size of colony	2-5 mm
Colour/Pigmentation	White and glistering
Elevation	Convex
Margin	Entire
Colony appearance	Opaque
Motility	Non motile
Bacterium shape	Small Rod shaped
Oxygen demand	Aerobic
Spore formation	Non spore forming
Gram Reaction	Gram negative

Table 5. Biochemical characteristics of *Burkholderia* isolates

Sr. No.	Test	Result
1	Starch hydrolysis	Positive
2	Catalase activity	Positive
3	Lipid hydrolysis	Negative
4	Citrate utilization	Positive
5	Casein hydrolysis	Positive
6	Gelatin liquefaction	Positive
7	Urease activity	Negative
8	H_2S production	Negative

Estimation of nitrogenase activity

Nitrogenase activity of the isolates was studied. The activity varied considerably among the *Burkholderia* isolates (60.65 to 63.45 n moles of ethylene produced/hr./ml). The maximum nitrogenase activity has been observed in B1 (63.45 n moles), while minimum nitrogenase activity has been recorded in isolate M2 (63.45 n moles) (Table 7 and Fig. 4). The maximum nitrogenase activity was observed in the B1, R1, M2 and S2. Regarding the nitrogenase activity it has been observed that *Burkholderia* isolates had variable activity of nitrogenase as induced in free living state. The free living forms of many *Burkholderia* strains when cultured in an appropriate medium exhibit nitrogenase activity. Similar findings were reported by many authors in different species of *Burkholderia*; *Burkholderia silvatlantica* sp. (Perin *et al*. 2006), *Burkholderia unamae* sp. (Mellado *et al*. 2004), *Burkholderia tropica* sp. (Reis *et al*. 2004).

Phosphate solubilization

Zone of 'P' hydrolysis formed by the test organisms in HAP plates was measured following incubation of 2-4 days. The isolate B1 formed better zone which measured 0.40 cm followed by isolate R1 (0.35cm) (Fig. 5 and Table 8). Ayyakkannu and Chandramohan (1971) studied phosphobacteria in marine environments, especially in sediments. Linu *et al*. (2009) studied phosphate solubilizing ability of *Burkholderia* sp. in interaction with cow pea. Park *et al*. (2010) reported the same result in case of novel environmental stress-tolerant *Burkholderia vietnamiensis* M6 isolated from ginseng rhizospheric soil.

Bio control activity

Antagonistic activity of *Burkholderia* was tested by dual culture method. Among the 10 isolates, B1 and R1 formed clear zone and inhibited the plant pathogen *Macrophomina phaseolina* and *Rhizoctonia solani* (Fig. 6 & Fig. 7). Zone

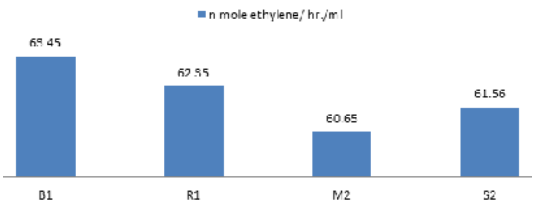


Fig. 4. Nitrogenase activity of *Burkholderia* isolates

Table 6. pH increase in BMGM broth by selected *Burkholderia* isolates

S. No.	Isolates	2nd day	4th day	7th day	9th day	13th day	17th day	20th day
1	Control	5.7	5.7	5.7	5.7	5.7	5.7	5.7
2	B1	7.8	8	8.4	8.5	8.6	8.9	9.01
3	R1	7.6	8	8.5	8.5	8.6	8.9	9.05
4	M1	5.9	6.2	6.5	6.5	6.6	7	7.22
5	M2	6.9	7	7.6	7.8	8	8.5	8.9
6	S1	7	7.3	7.8	8.1	8.3	8.5	8.63
7	S2	7.5	8	8.3	8.5	8.6	8.7	9.01
8	CH2	7.2	7.5	8	8.3	8.55	8.6	8.85
9	CO1	7	7.5	7.9	8	8.3	8.5	8.79
10	CO2	5.7	6	6.5	6.5	6.6	7.2	7.93

formed by B1 isolate in dual culture plate was measured as 2 cm and that of R1 is 1 cm. Rangeshwaran and Prasad (2000) reported rhizosphere bacteria showing antagonism against *Sclerotium rolfsii* the causal organism of root/collar rot in sunflower. Sfalanga *et al*. (1999) found new antagonistic *Burkholderia* strain from the rhizosphere of healthy tomato plants that was resistant to several antibiotic substances and suppress the growth of important bacterial and fungal phytopathogens. Ganyu *et al*. (2009) reported *Burkholderia* bacteria are frequently isolated from the rhizosphere of crops, and are involved in growth promotion of plants and suppression of plant diseases, *Burkholderia contaminans* strain MS14 has a broad range of antifungal activities to plant and human pathogens especially for *Geotrichum candidum*. These reports are in conformation with the findings of present investigation for antagonistic properties of *Burkholderia* against different plant pathogens.

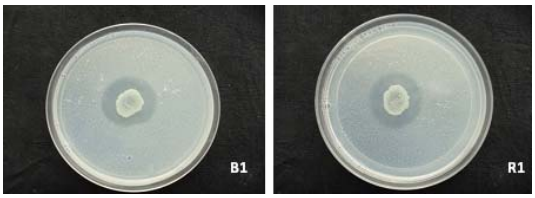


Fig. 5. Phosphate solubilization by *Burkholderia* sp.

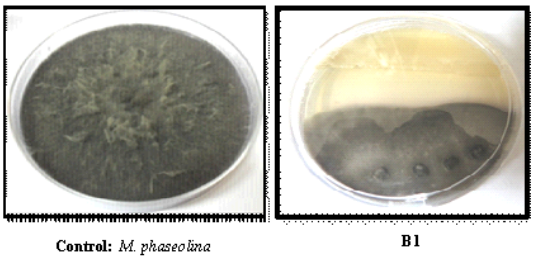


Fig. 6. Antagonistic activity of *Burkholderia* isolates against *M. phaseolina*

16S rRNA gene amplification of *Burkholderia* isolates

Amplification of the 16S rRNA gene of the four isolates was done by using primers mentioned earlier. When the 16S rRNA region of the each of the five isolates was amplified by PCR, a major amplification band of 1.5 kb was observed

Table 7. Nitrogenase activity of *Burkholderia* cultures.

Cultures	Nitrogenase activity(n moles of ethylene produced/hr./ml)
B1	63.45
R1	62.35
M2	60.65
S2	61.56

Table 8. Phosphate solubilization Zone formation by *Burkholderia*

Sr. No.	Name of isolate	Zone (in cm)
1	B1	0.40
2	R1	0.35
3	M2	0.34
4	S2	0.32
5	S1	0.18
6	C1	0.25
7	T1	0.30
8	T2	0.14
9	T3	0.22
10	BR1	0.16

and it was notified by PCR product on 0.8 per cent agarose gel (Fig. 8). Similar set of primers used by Marchesi *et al*. (1998) to amplify 16S rRNA genes from template DNA extracted from variety of organisms. Similar findings were reported by Gee *et al*. (2003) in *Burkholderia pseudomallei* and *B. mallei*. Taghavi *et al*. (1996) obtained similar result in *Burkholderia solanacearum, Pseudomonas syzygii*. In the Sulfide-oxidizing bacteria of the genus *Beggiato* Brock *et al*. (2012) and Ghosh *et al*. (2006) made similar observations. Yabuuchi *et al*. (1992) reported similar findings in *Burkholderia cepacia and* Genus *Pseudomonas.*

Nif H gene amplification of *Burkholderia* isolates

Amplification of the nif H gene of the 4 isolates was done by using the primers as mentioned earlier. When the nif H region of each of the four isolates was amplified by PCR, a major amplification band of 400 bp was observed, and it was notified by run the PCR product on 1 per cent agarose gel (Fig. 9). The nif H DNA region of *Burkholderia species* located, sequenced and identified the regulatory DNA elements involved in nif H expression by many authors. Nif D and Nif K genes *Burkholderia* endosymbiont of the arbuscular mycorrhizal fungus *Gigaspora margarita* reported by Minerdi *et al*. (2001). Twenty *Mimosa* nodulating bacterial strains isolated from Brazil and Venezuela reported to have nod A *and* nif H genes. (Chen *et al*. 2005).

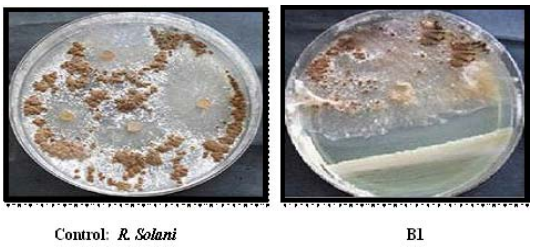
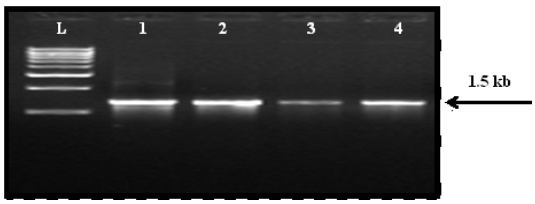
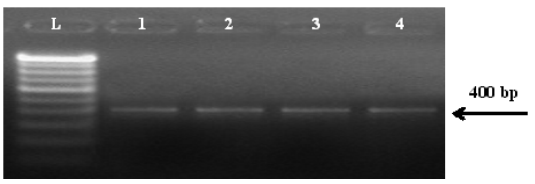


Fig. 7. Antagonistic activity of *Burkholderia* isolates against *R. Solani*



Legend:L-1kb ladder, 1-B1, 2-R1, 3-M2, 4-S2

Fig. 8. 16S rRNA gene amplification of *Burkholderi*a isolates



Legend:L-1kb ladder, 1-B1, 2-R1, 3-M2, 4-S2

Fig. 9. Nif H gene amplification in *Burkholderia* isolates

Sequencing Analysis

The amplified 16S rRNA gene PCR product of 2 isolates (B1 and R1) 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. Homology search of nucleotide sequences obtained from all four isolates with other reported 16S rRNA gene sequence was carried out individually. The 16S rRNA sequence of isolate B1 showed homology with *Burkholderia thailandensis* sp. 16S rRNA gene sequence. R1 showed homology with *Burkholderia vietnamiensis* 16S rRNA gene sequence. M2 shows homology with *Burkholderia ambifaria* 16S rRNA gene sequence. S2 shows homology with *Burkholderia vietnamiensis* 16S rRNA gene sequence.

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