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ORIGINAL ARTICLE

Characterization of a profenofos degrading bacterial isolate, *Pseudomonas aeruginosa* strain BRD1

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ABSTRACT
A significant profenofos (PFF) degrading bacterium, designated as BRD1, was isolated from an agricultural field of Purba Bardhaman district, West Bengal, India. The current study mainly focused on utilization of PFF as a sole carbon source, plant growth promoting activity, stress response and antibiotic sensitivity of the bacterium. The PFF degradative phenotype of BRD1 isolate was detected by thin layer chromatography (TLC). It was observed that the isolate Pseudomonas aeruginosa strain BRD1 is capable in producing HCN and exopolysaccharide (EPS) and can tolerate the heavy metal compounds such as $MnSO_4$*,* $ZnCl_2$*, and* $CoCl_2$ *moderately and* $FeCl_3$ *highly. The isolate shows resistance against chloramphenicol, rifampicin, and ampicillin antibiotics in antibiotic susceptibility assay. The results from this investigation reveals that strain BRD1 is capable of tolerating various environmental stresses and able in promotion of plant growth with sustainable bioremediation.*
Keywords: *Pseudomonas aeruginosa strain BRD1, TLC, PGP activity, Heavy metal tolerance, Antibiotic assay*

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INTRODUCTION

Profenofos is an organophosphate group insecticide and used mainly in cotton, maize, tobacco; sugar beet, soybeans, potatoes cultivation to control aphids, lygus bugs, cotton bollworms, tobacco budworms, leaf webber, cotton leaf-perforator, beet armyworm, whitefly, spider mites, caterpillars' pests [PTID Database: https:// www. lilab- ecust. cn/ ptid/ and 1]. Unknowingly and unintentionally, the farmers applied this insecticide in agricultural fields at more than required concentration. Thus, the soil, water, and air system are gradually saturated by this toxic pesticide and its residues. In various scientific reports [2, 3] depicted that PFF affected several non-target organisms due to excessive application. It was reported that PFF effects on pollinators, plants, rats, humans, soil microbial communities [4]. In comparison to physiochemical methods, microbial bioremediation method is an eco-friendly and cost-effective alternative and it cannot create further secondary pollution. Besides the microbial bioremediation, pesticide degrading microorganisms may also have various plant growth promoting traits and help in sustainable agriculture by bioremediation of toxic compounds and providing various plant growth promoting (PGP) factors to the growing plants.

Certain soil bacteria, algae, fungi, plants and insects can produce hydrogen cyanide (HCN), a broad spectrum volatile secondary metabolite [5, 6, 7, 8]. It has been reported that cyanogenic bacteria inhibit various pathogens like phytopathogen, fungi, weeds, insects, nematodes, termites and can promote plant growth and disease control by antibiotics, siderophore production, and phytohormone production [9]. Therefore, an HCN-producing bacterium can be used as biopesticide and offers an ecofriendly approach in sustainable agricultural management.

In biological aspect, bacterial exopolysaccharides (EPSs) protect their cells from extreme temperature, salinity, aridity, UV-rays, pH values, osmotic stress, phagocytosis, and chemical agents like antibiotics, heavy metals, and oxidants [10, 11, 12, 13, 14]. EPSs play a role in bacterial adhesion, aggregation, and biofilm formation of the biofilm matrix [15, 16, 17]. Generally, EPS are classified into two groups, homopolysaccharides and heteropolysaccharides. The homopolysaccharides are composed of glucose or fructose, and heteropolysaccharides contain glucose, fructose, galactose, mannose, rhamnose, fucose, N-acetylglucosamine, and uronic acids [18,19]. Extensive application of antibiotics in agricultural fields helps the soil microorganisms to adapt against these. The bacterial species either have their innate antibiotic resistance characters or may acquire these from other bacteria by gene transfer [20, 21, 22]. It was reported that *Pseudomonas aeruginosa* showed tolerance against essential heavy metals Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cr^{3+}, $Cd^{2+}$and Pb^{2+} [23].

The present study focuses on a PFF degrading bacterial culture, evaluating its various characteristics like PGP activity, stress response, and antibiotic sensitivity. The work indicates that the strain has multifunctional potentiality of diverged adaptability and bioremediation in sustainable agro-environmental management.

MATERIAL AND METHODS

Chemicals

Analytical grade profenofos (PFF) with purity ≥ 95.0 % (HPLC area %) and 4-bromo-2-chlorophenol (BCP) with purity ≥ 98.5% (GC area %) were purchased from Sigma-Aldrich (Merck).

Isolation of bacterium

The bacterium was isolated through enrichment culture technique. The media contains only mineral salts supplemented with 100 ppm PFF as sole source of carbon.

Study of profenofos utilization

In order to check the profenofos utilization as sole carbon source by the bacterial isolate BRD1, fresh MM agar plates supplemented with different concentration (25 - 250 ppm) of PFF were prepared and fresh bacterial culture BRD1 was streaked on the plates and incubated at 32˚ C for 24-48 hrs for growth.

Degradation study through TLC

For degradation study, the bacterial culture BRD1 was grown in 25 ml of MM broth medium supplemented with PFF as sole source of carbon in the orbital shaker incubator at 32˚ C for specific times (24 h, 48 h, 72 h, and 96 h). Control was set for each MM broth supplemented with PFF without bacterial culture.

In this assay, 24 h, 48 h, 72 h, and 96 h extracted degradative samples were used. The extraction process was carried out according to the reported standardized protocol [24]. For this, 10 µl of standard PFF, and BCP were loaded in first and second lane and 40 µl of each extracted sample (24 h, 48 h, 72 h, and 96 h) were loaded respectively in third, fourth, fifth and sixth lanes on TLC plate (Merck) and run in n-hexane: acetone: ethyl acetate (80:10:10) solvent system. The parent compound and its hydrolysed intermediates were detected as spots under UV light exposure and identified by comparing their Rf value to their authentic standards (from Sigma Aldrich).

Comparative growth profile

The comparative growth profile of the bacterium was carried out in three separate batch culture systems. One system had MM broth supplemented with 100 ppm profenofos as sole carbon source along with its control set. The second system had MM broth supplemented with 1% trisodium citrate as an easy sole carbon source along with a control set. The third system had tryptone soya broth (TSB). In each system, 50 µl of 0.6 OD_{620} bacterial inoculum was added and incubated at 32°C in the BOD shaker incubator with 120 rpm. After each 30 min interval, optical density was measured up to 100 h at 620 nm against their control set by the chlorometer.

PGP assay

The BRD1 bacterium was subjected to various plant growth promoting assays like HCN and exopolysaccharide production.

Hydrogen cyanide (HCN) production test

In hydrogen cyanide (HCN) production assay, 0.44% glycine supplemented King's medium B base (proteose peptone 20 g/L, dipotassium hydrogen phosphate 1.5 g/L, magnesium sulphate heptahydrate 1.5 g/L, agar 2% and pH 7.2 ± 0.2) was used. The isolated bacterium was streaked on glycine supplemented King's B medium. The picric acid solution (0.05% picric acid in 2% sodium carbonate) soaked Whatman No. 1 filter paper was placed inside the lid of the petri plates and made air tight by parafilm. The plates were incubated at 32 ˚C for 48 h. The transformation of yellow to reddish-brown colouration indicates HCN production [25].

Exopolysaccharide (EPS) production test

In order to check exopolysaccharide production, Congo red agar plate was used. The modified medium was composed with Luria-Bertani broth supplemented with 5% sucrose, 0.08% Congo red and 2% agar by following the procedure [26]. The bacterial isolate was streaked on plate and incubated for 5 days at 32 ˚C. The formation of blackish colonies indicates the presence of EPS.

Stress tolerance

Thermo-tolerance

Thermotolerant profile of the bacterium was performed to check optimum growth temperature. The BRD1 isolate was inoculated in nutrient broth (pH 7.4 ± 0.2) media and incubated at 4 °C, 20 °C, 32 °C, 40 °C, and 50 °C for 6 days with a control set. In the inoculation step, 20 µl of 0.6 optical density (OD_{620}) culture was inoculated and absorbances were measured at 620 nm [27, 28].

Salt tolerance

To check salinity tolerance, NaCl supplemented nutrient broth (pH 7.4 ± 0.2) media were used. Here 10 different gradient NaCl (1% to 10%) supplemented nutrient broth media were prepared. One control set was prepared with 1% NaCl. Then 20 µl of 0.6 optical density (OD) culture was inoculated in each set except the control and incubated at 32˚ C with 120 rpm speed in the BOD incubator shaker for 6 days. After proper incubation, salt tolerance property was examined by measuring the absorbance in the colorimeter [27].

Heavy metals tolerance

To check heavy metal tolerance level, the bacterium was subjected to different heavy metal supplemented media. Here, nutrient broth media was used with pH 6.8. The six heavy metals compounds, $ZnCl_2$ - 50 µg/ml, $MnSO_4$ – 500 µg/ml, $CoCl_2$ - 20 µg/ml, $HgCl_2$ - 120 µg/ml, $Pb(NO_3)_2$ - 500 µg/ml, $FeCl_3$ - 750 µg/ml were used at this particulate concentration in nutrient broth media [27, 29, 30]. Then, 20 µl of BRDI inoculum (0.6 OD_{620}) was added in each test tube and incubated for 6 days at 32°C. After incubation, the absorbance of each test was taken at 620 nm by colorimeter.

pH tolerance

The bacterium was grown in nutrient broth with different pH to check optimum growth pH. The different pH levels (pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0) were used with one control set. In each test media, 50 µl of inoculum was added and incubated for 24 h at 32°C. The absorbance of the growth was measured at 620 nm by colorimeter.

Antibiotic susceptibility assay

This test was performed to determine the susceptibility of the bacterial strain against the antibiotics. To conduct these tests, both the disc and cup method were applied in tryptone soya agar plate. A soft agar of 0.8% was used in the cup method to repair the bottom cracks of the wells.

In disc method, commercially purchased HiMedia antibiotic susceptibility disc like ciprofloxacin, levofloxacin, chloramphenicol, ampicillin, polymyxin-B, olfoxacin, erythromycin, norfloxacin, gatifloxacin, rifampicin discs were used and attached aseptically on the agar media in quadrant method and incubated at 32°C for 48 h.

In agar cup method, only tetracyclin, kanamycin and ampicillin were added at 200 ppm concentration into the wells and then incubated at 32°C for 48 h.

RESULTS AND DISCUSSION

Isolation of the bacterium

The BRD1 culture was isolated through enrichment culture technique where PFF (100 ppm) was used as a sole source of carbon. It was isolated from an agricultural field of Purba Bardhaman, West Bengal, India. In purification of the culture, the isolated bacterium was streaked on tryptone soya agar (TSA) plates for 5 times. The purified bacterium was maintained at both PFF enriched agar media (fig.1a), TSA plates (fig.1b) and slant (fig.1c) as well as in PFF broth (fig.1d). The isolated bacterium was identified as *Pseudomonas aeruginosa* strain BRD1 and possesses GenBank accession no- PV649922.

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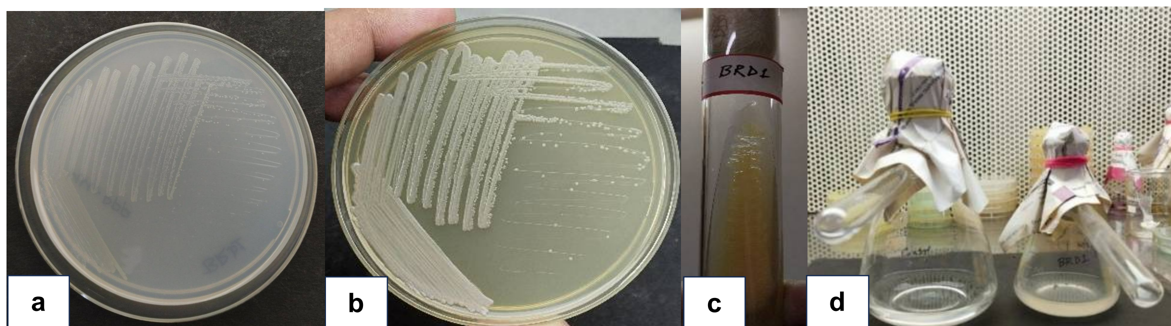

Fig. 1. (a) 24 h grown BRD1 culture in MM+PFF (100 ppm) plate; (b) Purified BRD1 culture in TSA plate; (c) Slant culture in TSA; and (d) 72 h BRD1 culture in MM+PFF (100 ppm) broth

Profenofos utilization pattern

In plate-based screening (25-250 ppm PFF), the BRD1 showed maximum growth in 100 ppm PFF plate in 48 h of incubation. But, among other concentrations, the culture showed relatively poor growth. The entire result was tabulated in table 1.

Table.1. Profenofos utilization profile of BRD1 isolate at different concentrations

Designation of the isolate	Different concentrations of PFF (ppm)									
	25	50	75	100	125	150	175	200	225	250
BRD1	+	++	++	++++	+++	++	++	++	++	+

"+" indicates poor utilization, "++" and "+++" relatively moderate utilization, and "++++" good utilization

Detection of PFF degradation by TLC

Under UV exposure, the TLC plate (fig.2) showed several spots. The larger spots of 24 h, 48 h, 72 h, and 96 h samples were compared with the spot of the BCP standards and it indicates the presence of BCP as a major hydrolytic intermediate of PFF in degradative samples. On the other hand, many small spots/zones were observed at run lanes (lane 3-6) of samples that indicate presence of other hydrolytic intermediates that cannot be identified by TLC.

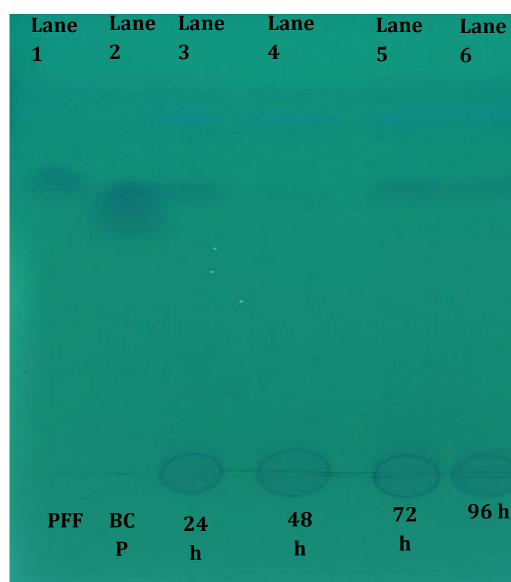

Fig.2. TLC assay for detection of profenofos biodegradation by *Pseudomonas aeruginosa* strain BRD1

Comparative growth profile

The 100 h growth studies of BRD1 in 3 different media, the BRD1 strain showed highest growth (1.95 OD_{620}) in tryptone soya broth (TSB), moderate (1.20 OD_{620}) in MM + citrate broth and very less (0.34 OD_{620}) in MM + PFF (100 ppm) broth. Critically, the BRD1 strain was entered into the stationary phase at 75 h, 62 h and 59 h respectively in MM + PFF, MM + citrate and TSB broth. The decline phase was started at 86 h, 78 h and 76 h respectively in MM + PFF, MM + citrate and TSB broth (Fig. 3).

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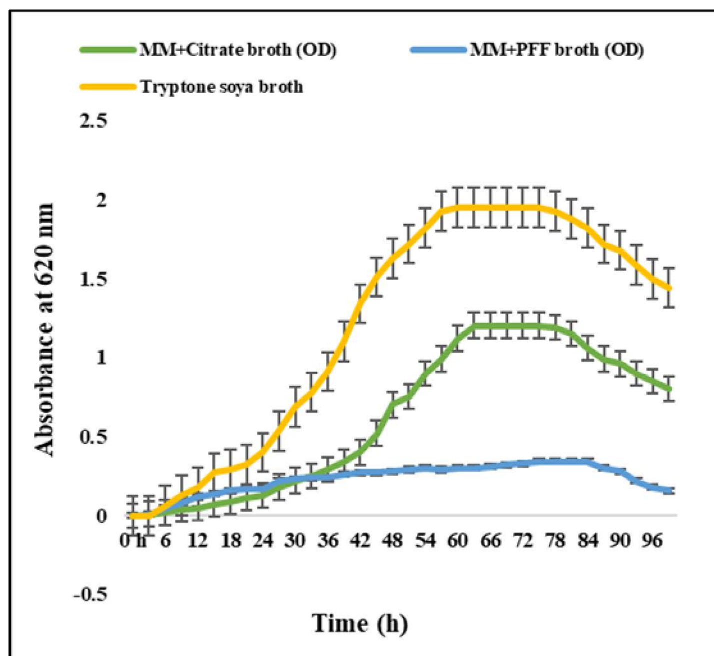

Fig. 3. Comparative growth profile of *Pseudomonas aeruginosa* strain BRD1 in MM + PFF (100 ppm), MM + Citrate (1%) and TSA media

PGP activity

In HCN production and exopolysaccharide secretion assay, the BRD1 strain showed positive response (fig.4) indicating that the strain can produce hydrogen cyanide, exopolysaccharide.

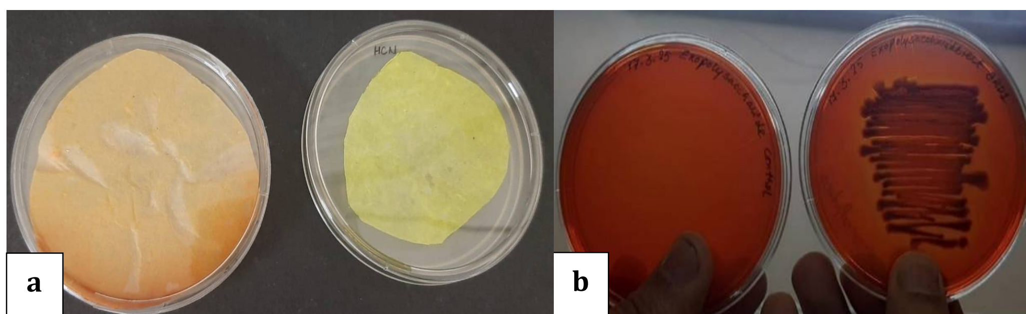

Fig.4. (a) HCN production test: left (test BRD1) and Right (Control); (b) Exopolysaccharide production test: left (control) and right (test BRD1)

Stress response characterization

During standardization of optimal growth temperature, the bacterium showed optimal growth at 32 °C (Fig. 5a). In salt response, BRD1 strain showed highest growth in 1% NaCl and can tolerate up to 4% and thereafter it shows decline of growth rate (Fig. 5b). In case of heavy metal tolerance assay, the strain BRD1 showed maximum growth rate in ferric chloride (1.01 OD_{620}), manganese sulphate (0.67 OD_{620}), zinc chloride (0.67 OD_{620}), cobalt chloride (0.5 OD_{620}), lead nitrate (0.3 OD_{620}) and lowest growth in mercuric chloride (0.07 OD_{620}) (Fig. 5c). In pH tolerance assay, the BRD1 isolate showed highest growth rate at the pH between 7 and 8 and moderately at pH 4 (Fig. 5d).

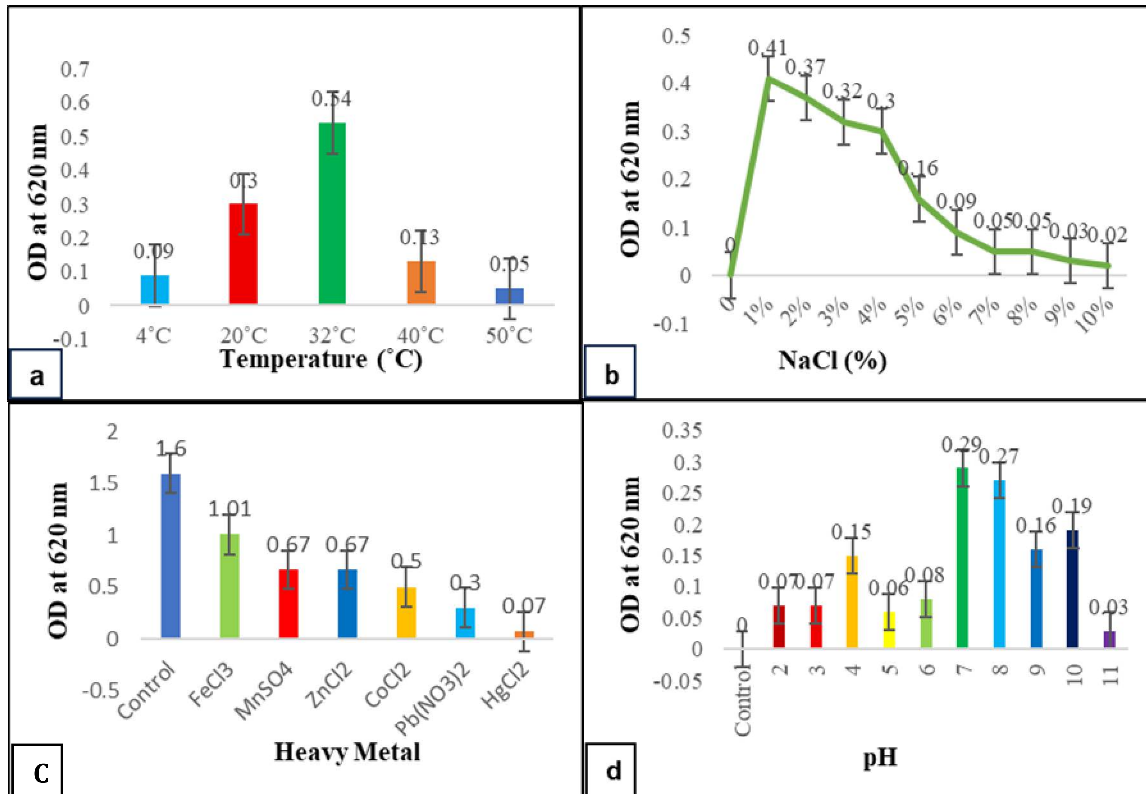

Fig. 5. Stress Response phenotype of BRD1 isolate: (a) Thermotolerance profile; (b) NaCl tolerance profile; (c) Heavy metals tolerance profile and (d) Effect of pH in growth

Antibiotic susceptibility response

The 48 hrs grown BRD1 culture showed cidal susceptibility against different antimicrobial agents like ciprofloxacin, levofloxacin, gatifloxacin, norfloxacin, polymyxin B, ofloxacin. Out of these, only ciprofloxacin, levofloxacin, norfloxacin, gatifloxacin and ofloxacin showed strong cidal effects (Fig. 6a, 6c, 6b), and erythromycin, kanamycin and tetracycline showed static effects on BRD1 isolate (Fig. 6b, 6d). The bacterium showed resistance to chloramphenicol, rifampicin and ampicillin antibiotics (Fig. 6a, 6c, 6d). The entire antibiotics susceptibility responses of *Pseudomonas aeruginosa* strain BRD1 was enlisted in Table 2.

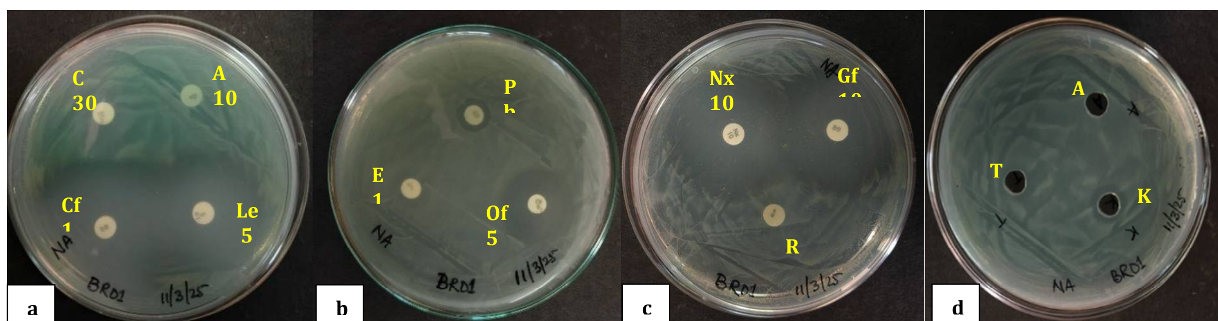

Fig. 6. Antibiotic susceptibility test of *Pseudomonas aeruginosa* strain BRD1: I. Disc Method (a, b, c): (**a**: Ciprofloxacin, Levofloxacin, Chloramphenicol, Ampicillin; **b**: Polymyxin B, Olfoxacin, Erythromycin; **c**: Norfloxacin, Gatifloxacin, Rifampicin); II. Agar Cup Method: (**d:** Ampicillin, Tetracycline, Kanamycin)

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Table. 2. Antibiotic Susceptibility assay of *Pseudomonas aeruginosa* strain BRD1

Sl. No.	Name of Antibiotics	Sensitive		Resistant
		Cidal	**Static**	
i	Ciprofloxacin	+	-	-
ii	Levofloxacin	+	-	-
iii	Chloramphenicol	-	-	+
iv	Rifampicin	-	-	+
v	Gatifloxacin	+	-	-
vi	Norfloxacin	+	-	-
vii	Polymyxin B	+	-	-
viii	Olfoxacin	+	-	-
ix	Erythromycin	-	+	-
x	Kanamycin	-	+	-
xi	Ampicillin	-	-	+
xii	Tetracycline	-	+	-

* '+' indicates positive and '-' indicates negative response in results

CONCLUSION

In this current study, the strain BRD1 possesses the HCN and EPS production and it can promote the plant growth. Beside this, the strain can also tolerate different heavy metals and possesses salt tolerance, antibiotic resistance characteristics. Therefore, *Pseudomonas aeruginosa* strain BRD1 may be considered as a biotechnologically important microorganism and suitable for various industrial applications, and bioremediation of various toxic compounds. As per its HCN, EPS production and antibiotic resistance characteristics, this culture can be used as a biopesticide for sustainable agricultural management and increment in crop production.

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