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FULL-LENGTH RESEARCH ARTICLE

Deciphering Rhizosphere Soil System for Strains Having Plant Growth Promoting and Bioremediation Traits

Keshav Prasad Shukla · Shivesh Sharma · Nand Kumar Singh · Vasudha Singh

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Abstract The rhizosphere is an important region of microbial interactions where exudates released by plant roots are the main source of food for microorganisms, which play a vital role in increasing their population density and activities. An isolate of bacterium *Pseudomonas* sp., designated as KS51, that expressed plant growth-promoting traits and antagonistic activity was isolated from the rhizospheric soil of *Calotropis* sp., grown in the northern and central regions of India. The isolate was recognized as Gram negative rod (0.67 × 2.89 µm) shaped bacterium. It grew at optimum temperature ranging from 25 to 30 °C, exhibited tolerance to a wide pH range of 6–10 and tolerated salt concentrations up to 5 % (wt/vol). Though, it was sensitive to chloramphenicol, ampicillin, tetracycline, gentamicin, ceftriaxone, and erythromycin, it showed resistance to co-trimoxazole, cefuroxime, ciprofloxacin, penicillin G, augmentin, fusidic acid and vancomycin. The bacterium was bio-chemically characterized and examined in vitro for their plant growth-promoting traits like production of indole acetic acid (IAA) (8 µg ml^{-1} day^{-1}), hydrogen cyanide (HCN), siderophore and phosphate solubilization (268 µg ml^{-1} after 144 h). The mean growth rate constant (*K*) of isolate was found to increase with successive increments in substrate concentration of naphthalene and anthracene (0.5–1.0 mg/50 ml). KS51 was also found to be a good degrader for naphthalene (78.44 %) and anthracene (63.53 %) as determined by HPLC analysis. Based on the 16S rRNA analysis, KS51 showed the maximum similarity with *Pseudomonas* sp. On the basis of its growth-promoting, biocontrol and bioremediation potential properties, KS51 could be applied in biotechnological applications.

Keywords Antagonistic activity · Bioremediation · Plant growth-promoting rhizobacteria (PGPR) · *Pseudomonas* · Rhizosphere

Introduction

The rhizosphere is an environment that the plant itself helps to create and where pathogenic and beneficial microorganisms constitute a major influential force on plant growth and health [13]. Many bacteria have a neutral effect on the plant, but are part of the complex food web that utilizes the large amount of carbon that is fixed by the plant and released into the rhizosphere (i.e. rhizodeposits) [10]. The bacterial community in the rhizosphere also harbours members that exert deleterious or beneficial effects on the plant. Bacteria that adversely affect plant growth and health are the pathogenic, whereas bacteria that are beneficial include nitrogen-fixing bacteria and plant growth-promoting rhizobacteria (PGPR) [34]. *Pseudomonas* sp. is one of the most important members of PGPRs showing all the three major group of PGPRs, that is having biofertilizer, phytostimulator and phytopathogen biocontrol activities [31]. Several *Pseudomonas* sp. have been extensively used for biological control against many soil borne plant pathogens [33].

It has been observed that polyaromatic hydro-carbons (PAH) degradation in soil is dominated by bacterial strains belonging to a very limited number of taxonomic groups such as *Sphingomonas*, *Burkholderia*, *Pseudomonas* [9].

K. P. Shukla · S. Sharma (✉) · N. K. Singh · V. Singh
Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad, UP, India
e-mail: ssnvsharma@gmail.com

Bacterial cell surface hydrophobicity is important in promoting cell adhesion to soil particles [23] and plays a critical role in facilitating biodegradation in situ [24]. However, the hydrophobicity and motility can potentially increase the ability of bacteria to access polycyclic aromatic hydrocarbons (PAHs) within soil [9]. Although, numerous studies have been conducted on *Pseudomonas* sp. and their possible role in plant growth promotion and other traits, the information regarding the association of *Pseudomonas* sp. in the rhizosphere niche of *Calotropis* plant possessing plant growth- promoting (PGP) traits, degradation abilities and hydrophobicity has not been yet been explored. Therefore, keeping in view the above constricts, the current study was designed to (i) isolate *Pseudomonas* sp. from the rhizosphere of *Calotropis* sp., growing in northern and central regions of India; (ii) screen the abilities of plant growth promoting attributes including the production of IAA hormone, siderophore, phosphate solubilization and the ability to inhibit pathogenic fungi *Alternaria alternata*, *Fusarium oxysporum* and *Cladosporium oxysporum* and (iii) screening with respect to ability to utilize PAHs, quantitative degradation by HPLC, growth study with varying concentration of anthracene and naphthalene and cell surface hydrophobicity of isolate. In addition, the morphological, physiological, biochemical and 16S rRNA analysis has been attempted to determine genus and species taxa of isolated strain.

Materials and Methods

Isolation

The soil used for bacterial isolation was collected from the rhizosphere of *Calotropis* sp. found to grow in four different zones of the northern and central India. The rhizospheric soil was collected in sterile polythene bags and stored at the 4 °C until further use. The pure culture was maintained on slants at 4 °C and in 10 % glycerol at 20 °C.

Identification and Characterization of the Bacterial Isolate

Phenotypic characterization of the isolate was carried out by subjecting the bacterial isolate to cultural (oxygen requirement), morphological (colony morphology and pigmentation), microscopic (Gram staining, cell shape, size and arrangement of cells), biochemical (utilization of different carbon sources and enzyme activity) and physiological characterization (temperature, pH, salt tolerance and antibiotic sensitivity) following standard procedures [7].

Qualitative and Quantitative Estimation of PGP Attributes

Salkowski's reagent was used to examine the indole acetic acid (IAA) production [19]. Quantitative estimation of IAA production was carried out by the standard method [8]. The amount of IAA produced in culture medium was calculated by comparing it with standard curve in μg/ml.

The potential to solubilize relatively insoluble calcium phosphate was checked by point inoculation on Pikovaskaya's agar and the plates were incubated at 28 °C for 4–5 days [25]. The zone was measured and phosphate-solublizing index (PSI) was calculated by means of the formula:

$$\mathrm{PSI} = \text{Colony diameter} + \text{Halozone diameter}/\text{Colony diameter}$$

Quantitative estimation of phosphate solubilization for selected isolate was carried out by chlorostanus-reduced molybdo-phosphoric acid blue method [29]. HCN production was checked by culturing the organism on nutrient agar medium supplemented with glycine (0.44 %). Filter paper dipped in 0.5 % picric acid was attached to the lid of culture tube. After incubation for 48 h at 28 ± 1 °C, colour change was observed for HCN production [4]. Siderophore production was detected qualitatively on Chrome-Azurol S agar (CAS) medium [21].

Growth Profile of Isolates in Anthracene or Naphthalene and HPLC Analysis

Growth profile of isolates in anthracene or naphthalene amended medium was also determined. Minimum salt basal medium was supplemented with different concentrations (0.5, 0.8 and 1.0 mg/50 ml) of anthracene or naphthalene. Mean growth rate (K) was calculated by formula given as follows:

$$K = 3.322\ \log Z_t - Z_0/\Delta T$$

where K is the mean growth rate constant, Z_t is final growth at time t, Z_0 is initial growth at time 0, and ΔT is difference in time.

Residual amounts of anthracene and naphthalene were determined by high performance liquid chromatography (HPLC) analysis in culture medium for quantitative estimation of PAH degradation.

Antagonistic Properties Due to Diffusible and Volatile Compounds

For examining the antagonism due to diffusible compounds produced by the bacterial isolate, fungal culture of test fungi (*F. oxysporum, A. alternata,* and *C. oxysporum*) were

individually inoculated on potato dextrose agar (PDA) plate. The inoculation of bacterial culture was made about 2 cm away from fungal inoculation. The plates were incubated in inverted position at 28 °C in the incubator. The percentage growth inhibition (PGI) was calculated using the formula:

$$\mathrm{PGI} = [(R_1 - R_2)/R_1 \times 100],$$

where R_1 represents the radius of the test fungus in the direction with no bacterial colony, and R_2 is the radius of the fungal colony in the direction of the bacterial colony. The antagonism due to volatile compounds was evaluated by preparing a bacterial lawn on nutrient agar plates. After incubation for 24 h, the lid was replaced by a plate containing an agar block of the test fungus grown on potato carrot agar (PCA). The two plates were sealed together with parafilm. Control sets were prepared in similar manner, without bacteria in the bottom plate. Such sealed sets of petri dishes were incubated at 28 °C. The PGI was calculated using the formula:

$$\mathrm{PGI} = [(R_1 - R_2)/R_1 \times 100]$$

where R_1 represents the radius of the test fungus in the control, and R_2 is the radius of the fungal colony with the bacterial colony [22].

Phylogenetic Analysis

Molecular characterization was carried out by 16S ribosomal RNA gene sequencing of the isolate. It was performed using universal eubacterial primers fD1 and rp2 [32]. The phylogenetic tree was constructed by the neighbour joining method using the distance matrix from the alignment [26].

Statistical Analysis

The data presented have been analysed statistically with the relevant standard deviation and standard error (SE) method.

Results

Isolation and Biochemical Characterization

On the basis of morphological and biochemical characterization, the bacterial isolate was found to be aerobic, pigmented (yellow), Gram-negative, rod-shaped and motile. It was also found that the isolate was positive for glucose fermentation, casein, gelatine, starch, methyl red, nitrate reduction, urea hydrolysis, catalase and oxidase and showed negative results for indole, voges proskauer, mannitol and citrate utilization test. However, it was found to be sensitive to chloramphenicol, ampicillin, tetracycline, gentamicin, ceftriaxone and erythromycin, and it showed resistance to co-trimoxazole, cefuroxime, ciprofloxacin, penicillin G, augmentin, fusidic acid and vancomycin (Table 1). The strain showed optimum growth between 25 and 30 °C, exhibited tolerance to a wide pH range between 6 and 10 and salt concentrations up to 5 % (wt/vol). On the basis of morphological, biochemical and physiological characteristics, the isolate was grouped under *Pseudomonas* sp. as described in Bergey's Manual of Determinative Bacteriology.

Plant Growth Promoting Traits of Test Isolates

After 24, 48 and 72 h incubation time, the isolate was able to produce 8, 13 and 18 μg ml^{-1} day^{-1}, IAA, respectively (Table 2). It was interesting to observe that the isolate was able to retain its functional traits even after 72 h. Phosphate solubilization was evidently visible on the Pikovaskya agar plate where isolate form a clear halo zone around it. The zone of solubilization around the bacterial colony was found to increase with the increase of incubation time. Quantitative estimation of phosphate solubilization, estimated after incubation for duration ranging from 24 to 144 h, is presented in Fig. 1a. The bacteria solubilized 71 μg ml^{-1} of phosphorus on 24 h of incubation, and maximum solubilization (268 μg ml^{-1} of P) was recorded after 144 h. The pH of the phosphate solubilization in broth was found to decline because of bacterial activity and lowering of pH coincided with increase in the efficiency of phosphate-solubilizing activity. The pH was found to decline from 6.3 to 4.5 (Fig. 1b).

Formation of an orange halo zone around bacterial colony on Chrome azurole S agar medium indicates the siderophore production. A moderate siderophore production was witnessed after 24 h, and strong orange halo zones were observed after 48 and 72 h. However, the test isolates was found negative for HCN production (Table 2).

Growth Profile and HPLC Analysis

It was found that the growth rate constant increased with the increase in concentration of substrate. The K values of the isolate KS51 in medium amended with 0.5, 0.8 and 1 mg/50 ml anthracene was obtained as 0.33, 0.38 and 0.46 h^{-1}, respectively. Similar results were also obtained for 0.5, 0.8,s and 1 mg/50 ml naphthalene, where K values was found to be 0.26, 0.37 and 0.44 h^{-1}, respectively (Fig. 2).

The isolate was found to considerably reduce PAH concentration in medium as estimated by HPLC analysis. The isolate KS51 showed 63.53 % degradation of anthracene,

Table 1 Morphological, biochemical and physiological characteristics of *Pseudomonas* sp. isolate KS51

Morphological characteristics
Colony morphology and microscopic features: entire, circular, convex, translucent with smooth surface, yellowish; Gram negative, single, motile rods (0.67 × 2.89 μm in size)
Biochemical properties
Positive for glucose fermentation, casein, gelatine, starch, methyl red, nitrate reduction, urea hydrolysis, catalase and oxidase; negative for indole, voges proskauer tests, citrate utilization and mannitol.
Physiological characteristics
Temperature tolerance 4–35 °C, Optimum temperature 25 °C; pH tolerance 4–12, optimum pH 8.0; salt tolerance up to 4 % (w/v); antibiotic sensitivity (μg/ml) to chloramphenicol (30 μg ml^{-1}), ampicillin (10 μg ml^{-1}), tetracycline (30 μg ml^{-1}), gentamicin (10 μg ml^{-1}), ceftriaxone (30 μg ml^{-1}) and erythromycin (25 μg ml^{-1}); showed resistance to co-trimoxazole(25 μg ml^{-1}), cefuroxime (30 μg ml^{-1}), ciprofloxacin (5 μg ml^{-1}), penicillin G (30 μg ml^{-1}), augmentin (30 μg ml^{-1}), fusidic acid (30 μg ml^{-1}) and vancomycin (5 μg ml^{-1}).

Table 2 Plant growth-promoting attribute of *Pseudomonas* sp. at different incubation time

Incubation time (h)	PSI	P solubilization (μg/ml)	IAA production (μg/ml)	HCN Production	Siderophore
24	3.5 ± 0.32	71 ± 2.60	8 ± 0.17	–	++
48	4.13 ± .03	120 ± 3.60	13 ± 0.17	–	+++
72	4.53 ± .03	175 ± 4.58	18 ± 0.50	–	+++

Numerical values are mean ± SD of three independent observations

IAA indole acetic acid, *hcn* hydrogen cyanide. None, −; weak, +; moderate, ++; strong, +++; very strong

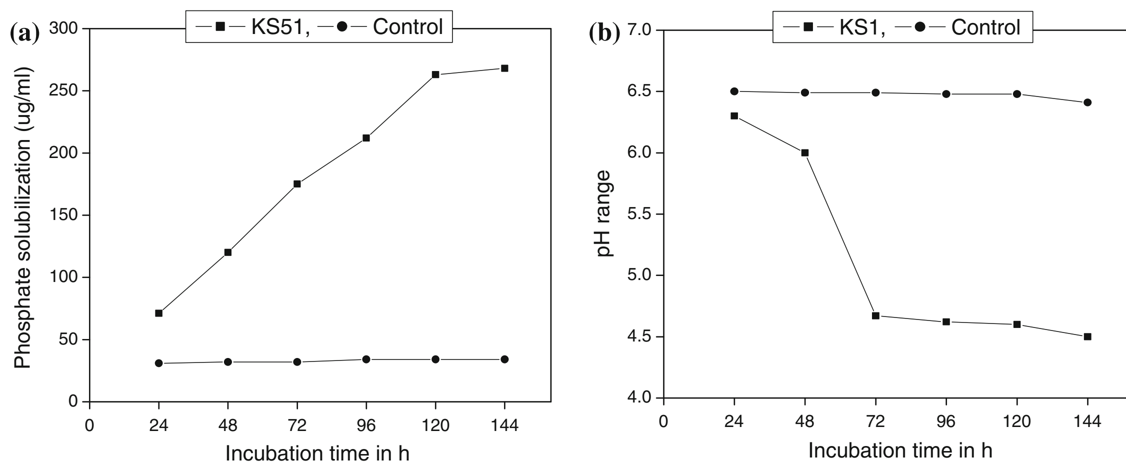

Fig. 1 **a** Quantitative P-solubilization by *Pseudomonas* sp. **b** Lowering of pH in the broth due to P-solubilizing activity of *Pseudomonas* sp. (*filled square* KS51 and *filled circle* for control)

while 78.44 % decrease in naphthalene concentration was recorded after 6 days.

Antifungal Activity of the Test Isolates

Biocontrol activity of the isolate was checked against *F. oxysporum*, *A. alternata* and *C. oxysporum*. The test isolate showed good antifungal activity fungi through the production of volatile metabolite but relatively less antifungal activity was detected through the diffusible metabolite (Table 3).

Phylogenetic Analysis

Based on the 16S ribosomal RNA gene sequence, isolate KS51 showed maximum similarity with *Pseudomonas* sp. (Accession No. FM173664.1). The phylogenetic tree (Fig. 3) constructed using 16S rRNA gene sequences of

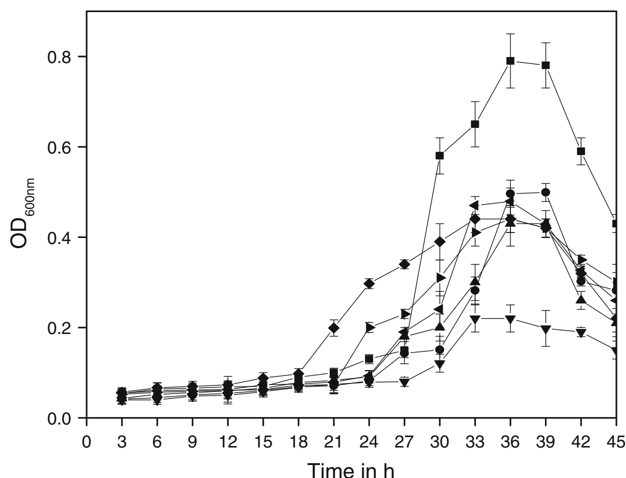

Fig. 2 Growth profile of isolate KS51 in medium supplemented with different concentrations of anthracene or naphthalene. Control (*filled square*), 0.5 mg/50 ml Ant (*filled circle*), 0.8 mg/50 ml Ant (*up-pointing triangle*), 1 mg/50 ml Ant (*down-pointing triangle*), 0.5 mg/50 ml Nap (*left- and right-pointing triangles*), 0.8 mg/50 ml Nap (*left-pointing triangles*), 1 mg/50 ml Nap (*right-pointing triangles*)

other related members of the *Pseudomonad* group revealed that the isolate formed a close cluster with *Pseudomonas xanthomarina* (Accession No. HQ202840.1).

Discussion

Rhizosphere is a rich source of microbes and should be explored for obtaining potential PGPRs, which might be good bio-inoculants for improving yields of crop plants. The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include (i) the ability to produce or change the concentration of plant growth regulators like IAA, gibberellic acid, cytokinins and ethylene; (ii) asymbiotic N_2 fixation; (iii) antagonism against phytopathogenic microorganisms by production of siderophores, antibiotics and cyanide; and (iv) solubilization of mineral phosphates and other nutrients [1]. In the present investigation, isolate KS51 of *Pseudomonas* sp. was screened in vitro for its PGP activities. Though the results of the present investigations are in conformity with earlier recorded observations, based on several species of *Pseudomonas* that produce secondary metabolites, mobilize nutrients and promote plant growth [31].

Production of phytohormone like auxin (IAA) is a desirable characteristic of a PGPR [20]. In the current investigation, IAA production was detected by the test isolate. Our findings of IAA production by the *Pseudomonas* isolate are in agreement with other researchers [11]. There was an increase in the concentration of IAA with the increasing incubation time. Similar trend of IAA production with the increasing concentration of incubation time has also been reported [27]. Such findings may have direct practical application, although intrinsic ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant [3].

The ability of the isolate to solubilize tricalcium phosphate in vitro is another important means of achieving plant growth promotion [14]. From the current study, it was evident that the isolate liberated maximum 268 μg/ml of available phosphorus after 144 h and the pH declined from 6.3 to 4.5. These results draws support from an earlier study which highlights production of a variety of organic acids by ten bacterial and three fungal strains and subsequent drop in pH [16]. The mechanism(s) of microbial solubilization of insoluble phosphate has received attention, and phosphate solubilization is considered an important attribute of plant growth-promoting rhizobacteria. Detailed studies including phylogenetic relationships on strains of phosphate-solubilizing *Pseudomonas* sp. isolated from different ecological niches have also been carried out [17].

Siderophore is one of the biocontrol mechanisms belonging to PGPR groups, and PGPRs produce a range of siderophore which have a very high affinity for iron [31]. Therefore, the low availability of iron in the environment would suppress the growth of pathogenic organisms including plant pathogenic fungi [33]. From this study, it was found that the isolate was a very good siderophore producer while the isolate displayed no HCN production.

In plant disease management programmes, the use of a rapid method for screening efficient biocontrol agents is a prerequisite [2]. Antagonistic activity possessed by biocontrol agents is often evaluated by measuring the inhibition zones developed under in vitro agar based assays [30]. In the present study, isolate was examined for its

Table 3 In vitro effects of diffusible and volatile metabolites of *Pseudomonas* sp. of *F. oxysporum*, *A. alternata* and *C. oxysporum*

% Growth inhibition *F. oxysporum*		% Growth inhibition *A. alternata*		% Growth inhibition *C. oxysporum*	
Diffusible	Volatile	Diffusible	Volatile	Diffusible	Volatile
40.00 ± 4.23	42.85 ± 1.80	36.89 ± 3.60	70.37 ± 5.00	21.34 ± 1.52	65.00 ± 3.00

Numerical values are mean ± SD of three independent observations

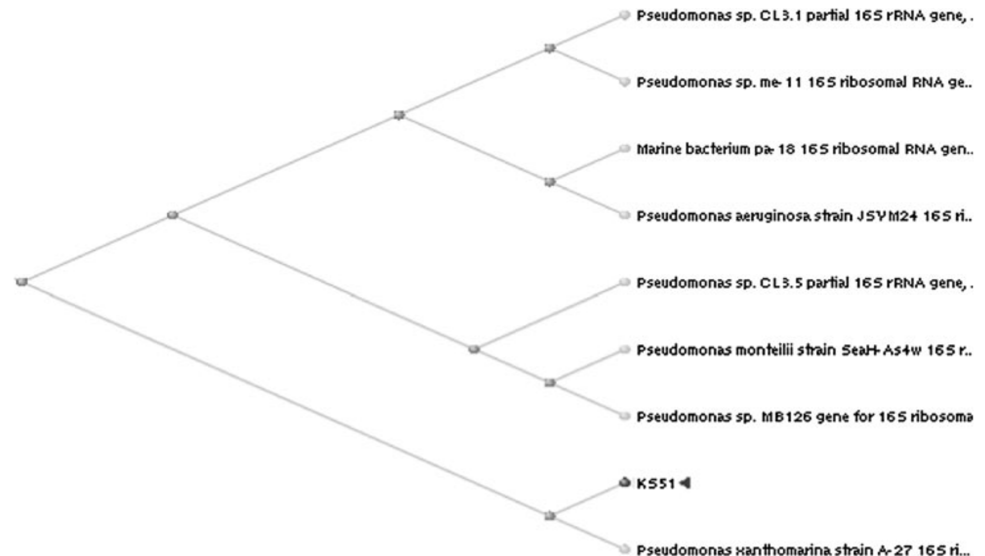

Fig. 3 Phylogenetic tree, based on 16S ribosomal RNA gene sequences, showing relationships between KS51 and other Pseudomonads

antagonistic activities against three phytopathogenic fungi, viz., *Alternaria alternata*, *Fusarium oxysporum* and *C. oxysporum*, and it showed inhibition in fungal growth (40 % in *F.* o*xysporum* and 36 % in *A.* a*lternata*) because of diffusible metabolites, while the maximum inhibition in fungal growth (70.37 % in *A. alternata* and 65 % in *C. oxysporum*) due to volatile metabolite was observed after 120 h of incubation. Similar results on the effectiveness of *Pseudomonas corrugata* against plant pathogenic fungi like *A. alternata and F. oxysporum* [30], *Fusarium*, *Rhizoctonia*, *Sclerotium*, *Pythium* [18] were also reported. Our results are in conformity with the studies of Trivedy et al. [30] which prove that the effect(s) of inhibitory volatile metabolite(s) produced by *P. corrugata* had a predominant inhibitory role in the antagonism of the test fungi, *A. alternata* and *F. oxysporum*, and the diffusible metabolite(s) played only a subsidiary role in the antagonism. In addition, some other studies are in contrast with the current study where the effect of inhibitory volatile metabolite(s) received less importance than the inhibitory diffusible metabolite(s) [28].

The growth rate of the isolate was found to increase with increase of substrate concentration. This further confirmed that anthracene and naphthalene concentrations are significant in growth physiology of the isolates and may also act as limiting nutrients in rhizosphere [6]. However, in this study, the quantitative estimation of residual amount of PAH in culture medium showed that 78.44 % of anthracene was degraded by the isolate [15] have also reported the degradation of PAH in liquid and solid medium by *Pseudomonas* sp. in their study. In the present study, the bacterium was isolated from the rhizosphere of *Calotropis* sp., found to grow in north and central part of India. They are highly prone to grow in extreme environmental conditions with temperature 0–50 °C, low rainfall 5–10 ml/annum, low relative humidity, high salinity and deposition of kankar or hardpan in the soil [12]. Therefore, it is assumed that the isolated bacterium has naturally adapted to variable extremities of temperatures and would retain its beneficial plant growth-promoting traits when inoculated in similar conditions.

The phylogenetic tree constructed on the 16S rRNA gene sequence revealed its close identity with *Pseudomonas* sp., a Gram-negative bacterium, which was first described from the rhizosphere of grasses [5]. The study carried by [31] support the present investigation to screen the *Pseudomonas* sp. isolated from rhizosphere of Soybean plant as plant growth promoter and biocontrol agent on the basis of 16S rRNA gene sequence analysis.

Thus, it can be concluded that rhizosphere of *Calotropis* sp. is a source of *Pseudomonas* sp. possessing potent PGP attributes, PAH degradation and biocontrol activities against phytopathogenic fungi. Further studies are underway to confirm their effectiveness in field conditions.

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