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ISOLATION OF POLYCYCLIC AROMATIC HYDROCARBONS DEGRADING PGPR FROM A OIL PRODUCTS CONTAMINATED SITE IN HYDERABAD, TELANGANA STATE, INDIA

M. POORNACHANDER RAO[1], A.B. RAJITHASRI*[2], K.SAGAR[3] AND K. SATYAPRASAD[4]

[1]Government Degree College, Mahabubabad, Waranagal (Dist.), Telangana, India
[2, 3,4]Mycology and Plant Pathology Laboratory, Department of Botany , Osmania Univesity, Hyderabad, Telangana, India

ABSTRACT

Rhizoremediation of polycyclic aromatic hydrocarbons (PAHs) with aid of Plant Growth Promoting Rhizobacteria (PGPR) is a novel, effective and emerging method in advance research of green chemistry. In the present study, the authors have isolated 26 PAHs degrading bacteria from PAHs contaminated soil samples of plants growing in premises of oil products manufacturing industries located in Cherlapally, Hyderabad. Depending upon growth of the isolates in Minimal salt medium (MSM) enriched with test PAHs phenanthrene, anthracene and pyrene effective PAHs degrading bacteria were identified. The same isolates were screened for the production of PGPR traits such as Indole acetic acid, hydrogen cyanide, ammonia and enzymes like phosphatases, proteases, cellulases, chitinases etc. Based on PAHs degradation and PGPR tests, the most effective PAHs degrading PGPR strain P14 was selected for further work. This isolate is gram positive, undulate, spore forming bacillus and biochemically positive for the reactions of ornithine utilization, nitrate reduction, citrate utilization, Voges Proskaure's test, methyl red test, cytochrome oxidase, catalase. This strain utilized sugars like glucose, lactose, saccharose, mellibiose, raffinose and trehalose on the characterization. In molecular characterization with 16rDNA sequencing the strain has showed maximum similarity with *Bacillus cereus*. Based upon all the characterization methods we named it as *Bacillus cereus* CPOU13.

KEY WORDS: Rhizoremediation, Polycyclic Aromatic Hydrocarbons, PGPR, Minimal Salt Medium, *Bacillus cereus.*

A.B. RAJITHASRI
Mycology and Plant Pathology Laboratory, Department of Botany , OsmaniaUnivesity, Hyderabad, Telangana, India

***Corresponding author**

INTRODUCTION

Presence of polycyclic aromatic hydrocarbons (PAHs) in the environment at high levels and due to their ubiquitous, carcinogenic, mutagenic, teratogenic and phytotoxic properties enforced the scientists to concentrate on remediation of PAHs[1]. Remediation for the removal of PAHs from the contaminated sites at the earliest is necessary to avoid deleterious effects on the environment and life forms like animals, humans beneficial microbes and plants. Bioremediation has become important in recent years due to its low cost, effectiveness, restoration of medium where it is operated and possesses several advantages. Microorganisms are the key participants as they have suitable enzymatic and physical properties for effective bioremediation. In this context rhizosphere associated bacteria effectively used to degrade organic pollutants in soil and extensively reviewed[2]. Plant growth promoting rhizobacteria (PGPR) are one of the important components of rhizosphere microflora. Kloepper and Schroth[3], (1978) coined the term plant growth promoting rhizobacteria (PGPR) to designate the beneficial rhizobacteria which improve crop productivity and growth by stimulating plant growth or by reducing the damage from soil-borne plant pathogens[4]. The direct participation by PGPR stimulate the plant growth, increase biomass and their indirect involvement provide beneficial qualities like biocontrol and inhibition of pathogens. For instance, PGPR produce plant growth regulators like Indole Acetic Acid (IAA), Gibberellins, Cytokinins and enzymes like phosphatases, nitrogenases that contribute to plant growth enhancement. Studies have been focused on PGPR as candidates for good PAHs remediation and improving remediation to several folds than the utilization of general PAHs degraders[5]. Isolation, identification of bacteria from the contaminated sites and assessment of the ability of rhizobacteria for growth promotion and to degrade the PAHs has been taken up in this study with the following objectives.

MATERIALS AND METHODS

Isolation of polycyclic aromatic hydrocarbons (PAHs) degrading rhizobacteria

Rhizosphere soils of plants growing on fly ash were collected as described by Bashan et al[6]. The method of enrichment and isolation of PAHs degrading bacteria was adopted from Dean-Ross et al[7]. The isolates were purified according to the procedure of Kiyohara et al[8]. The ability of the isolate to utilize PAHs was tested using three test PAHs such as phenanthrene, anthracene and pyrene following the method of John et al[9].

Screening for Plant Growth Promoting (PGP) traits

The PAHs degrading rhizobacterial isolates were assessed for their ability to produce plant growth promoting substances like ammonia, IAA, HCN and extracellular enzymes. Production of ammonia was assessed as described by Cappuccino and Sherman[10], auxin by the procedure of Gordon and Paleg[11], HCN by the method of Bakker and Schipper[12], chitinase was screened by the method of Rodriguez-Kabana[13], phosphatases by observing phosphate solubilization as described by Goldstein[14]. Quantification of phosphatase activity was done by adopting the method of Banerjee et al[15]. Production of proteases was screened on skim milk agar medium[16], cellulase enzymes was done on M 9 medium amended with carboxy methyl cellulose.

Characterization of selected isolates

Morphological characteristics of selected bacterial isolates such as colony, motility, spore formation and reaction with Gram stains were recorded as per standard laboratory manuals. Biochemical characteristics such as production of different enzymes on specified media and utilization of different carbohydrates were determined using 'Biochemical Characterization Kit' KB003 Hi 25 of Himedia. Molecular characterization with sequencing 16S rDNA gene for PAHs degrading

bacteria was adopted from Wang et al[17]. Phylogenetic tree was constructed for selected isolate by retrieving the bacterial strain names having the highest scores of percentage similarity and Neighbor-joining method[18]. The 16S rDNA sequences were submitted to NCBI GenBank by MyBaklt tool and got the accession number.

RESULTS

Isolation of polycyclic aromatic hydrocarbons (PAHs) degrading bacteria from rhizosphere soil

Rhizobacteria degrading polycyclic aromatic hydrocarbons (PAHs) were isolated from rhizosphere soil samples of plants growing in the premises of oil products manufacturing industrial premises located in Cherlapally, Hyderabad, Telangana State, India. Rhizosphere soils were collected from *Tridax procumbens*, *Cynodon dactylon*, *Chlorophytum laxum* etc. growing in the polluted area. In the present study, 26 PAHs degrading rhizobacteria were isolated on MSM broth enriched with test PAHs such as phenanthrene, anthracene or pyrene. The list of rhizobacterial isolates and their nature from the site is presented in Table 1.

Table 1
Characterstics of Rhizobacterial isolates from the samples of IOCL, HPCL premises on MSM

Isolate Name	Gram stain	Shape	Isolate Name	Gram stain	Shape
P 1	Gram positive	Coccus	P 14 ,	Gram positive	Bacillus
P2	Gram positive	Coccus	P 15	Gram positive	Bacillus
P 3	Gram positive	Bacillus	P 16	Gram positive	Bacillus
P4	Gram negative	Coccus	P17	Gram positive	Coccus
P 5	Gram positive	Coccus	P 18	Gram positive	Coccus
P 6	Gram positive	Bacillus	P 19	Gram negative	Bacillus
P 7	Gram negative	Bacillus	P 20	Gram positive	Bacillus
P 8	Gram positive	Bacillus	P 21	Gram negative	Bacillus
P 9	Gram positive	Coccus	P 22	Gram positive	Bacillus
P 10	Gram positive	Coccus	P 23	Gram positive	Coccus
P 11	Gram positive	Bacillus	P 24	Gram negative	Bacillus
P 12	Gram negative	Bacillus	P 25	Gram positive	Bacillus
P 13	Gram positive	Coccus	P 26	Gram positive	Bacillus

Screening rhizobacterial isolates for phenanthrene, anthracene and pyrene degradation

All the 26 isolated PAHs degrading rhizobacteria screened for their ability to grow on MSM enriched with phenanthrene, anthracene or pyrene (100ppm) as sole source of carbon, growth was recorded and the results are presented in Table 2. The rhizobacterial isolates P4, P13 and P14 showed good growth on MSM enriched with phenanthrene. While isolates P5, P7, P8, P9, P13, P14 and P24 showed good growth on MSM enriched with anthracene. Only P14 strain have shown best growth on MSM enriched with pyrene. Among the 26 isolates, one strain, P14 exhibited relatively best growth on phenanthrene, anthracene or pyrene enriched MSM. The PAHs degradation results suggest that almost all bacterial isolates have the ability to utilize all the three PAH compounds *viz*., phenanthrene, anthracene and pyrene as a source of carbon.

Table 2
Screening of rhizobacterial isolates for PAHs degradation on MSM broth enriched separately with phenanthrene, anthracene or pyrene (100 ppm)

Sl. No.	Isolate Name	Phenanthrene	Anthracene	Pyrene
1	Control	0	0	0
2	P 1	0.01	0.03	0.06
3	P 2	0.01	0.04	0.03
4	P 3	0.02	0.003	0.02
5	P 4	0.04	0.06	0.003
6	P 5	0.001	0.17	0.02
7	P 6	0.001	0.04	0.01
8	P 7	0.01	0.31	0.05
9	P 8	0.01	0.36	0.02
10	P 9	0.002	0.11	0.003
11	P 10	0.007	0.04	0.04
12	P 11	0.007	0.06	0.02
13	P 12	0.006	0.06	0.004
14	P 13	0.12	0.29	0.02
15	P 14	0.54	0.49	0.22
16	P 15	0.01	0.07	0.002
17	P 16	0.007	0.03	0.006
18	P 17	0.01	0.03	0.01
19	P 18	0.01	0.001	0.03
20	P 19	0.001	0.02	0.001
21	P 20	0.01	0.02	0.03
22	P 21	0.009	0.002	0.03
23	P 22	0.02	0.01	0.01
24	P 23	0.002	0.001	0.01
25	P 24	0.002	0.17	0.01
26	P 25	0.008	0.03	0.004
27	P 26	0.008	0.02	0.06

Screening for Plant Growth Promoting (PGP) traits by PAHs degrading rhizobacteria

Rhizobacterial isolates were screened for their ability to produce plant growth promoting traits qualitatively and quantitatively using specific media (Table 3). Among the 26 PAHs degrading bacterial isolates, 12 isolates *viz*., P3, P4, P6, P8, P9, P11, P13, P14, P19, P21, P23 and P24 were the best producers of ammonia while 12 other isolates produced ammonia moderately. Rest of the isolates (2) failed to produce ammonia. HCN production was assessed by the change of colour of picric acid containing paper from yellow to orange. As a slight colour change from yellow was considered as an indication for weak production of HCN in determining the ability of isolates. Among the isolated bacteria only 7 isolates, P12, P14, P15, P19, P20, P21 and P24 produced HCN moderately and 5 isolates showed low production. Out of 26 bacterial isolates 19 isolates produced the plant growth hormone ranging IAA from 3.5 to 86 µg/ml. Among these 19 isolates only 12 isolates did not produce IAA at all,, P14 produced high amount (86µg/ml) of IAA. In contrast, isolates P3, P11, P17, and P26 produced minimum quantity ranging from 1.5µg/ml to 3.5µg/ml. Others produced in moderate range from 5.5µg/ml to 8.5µg/ml.

Table 3
Production of plant growth promoting substances such as ammonia, IAA and HCN by the PAH degrading rhizobacterial isolates

Sl. No.	Isolate	Ammonia Production	IAA (µg/ml)	HCN Production
1	P 1	+	0	-
2	P 2	+	0	-
3	P 3	++	3.5	-
4	P 4	++	0	+
5	P 5	+	0	-
6	P 6	++	0	-
7	P 7	+	0	-
8	P 8	++	7	-
9	P 9	++	12	-
10	P 10	+	5.5	+
11	P 11	++	1.5	+
12	P 12	+	5.5	++
13	P 13	++	5.5	-
14	P 14	+++	86	++
15	P 15	+	7	++
16	P 16	+	5.5	-
17	P 17	+	3.5	-
18	P 18	-	7	+
19	P 19	++	7	++
20	P 20	-	8.5	++
21	P 21	++	7	++
22	P 22	+	7	+
23	P 23	++	7	+
24	P 24	++	0	++
25	P 25	+	7	+
26	P 26	+	3.5	-

- = No production; + = Low production; ++ = Moderate production; +++ = High production

Production of plant growth promoting/extra cellular enzymes by rhizobacterial isolates

In the screening for extracellular enzymes, the isolates P8, P12, P14, P18 and R 01 showed high (≥30 units) phosphatase activity followed by ten other isolates viz., P2, P4, P7, P8, P9, P15, P16, P19 and P20 which showed moderate phosphatase activity (20-30 units). High protease activity (≥3 units) was shown by P4, P6, P7, P10, P14, P19, P20, P21, P25 and P26, isolates, P3, P16 and P22 showed moderate activity (2-3 units). While P2, P8, P9, P13 and P15 isolates showed low protease activity. Low production of chitinase was observed among the isolates P7, P14 and P19 while other isolates failed to produce chitinase at all. No rhizobacterial isolate except P9 produced the enzyme cellulase. Rhizobacterial isolates, P3, P4, P7, P8, P9, P14, P15, P16, P19, P20, P21 and P25 showed production of protease and phosphatase. Among them, P4, P7, P14, P19 and P20 showed best production of protease and phosphatase. Protease, phosphatase and cellulase production was observed in the strain P9, while protease, phosphatase and chitinase production was found in the strains, P7, P14 and P19. The results for production of extracellular enzymes is showed in Table 4.

Table 4
Production of extracellular enzymes by PAHs degrading rhizobacterial isolates

S. No.	Isolate	Phosphatase		Protease		Cellulase	Chitinase
		Qualitative Test	Quantity (Units/ml)	Qualitative Test	Quantity (Units/ml)	Qualitative Test	Qualitative Test
1	P 01	-	0	-	0	-	-
2	P 2	++	21	+	1.24	-	-
3	P 3	+	11	++	2.65	-	-
4	P 4	++	25	+++	3.65	-	-
5	P 5	-	0	-	0	-	-
6	P 6	+	16	+++	3.98	-	-
7	P 7	++	18	+++	3.46	-	+
8	P 8	+++	35	+	1.74	-	-
9	P 9	++	22	+	1.68	+	-
10	P10	-	0	+++	3.69	-	-
11	P 11	-	0	-	0	-	-
12	P 12	+++	32	-	0	-	-
13	P 13	-	0	+	1.53	-	-
14	P 14	+++	55	+++	5.32	-	+
15	P 15	+	13	+	2.37	-	-
16	P 16	++	28	++	2.14	-	-
17	P 17	-	0	-	0	-	-
18	P 18	++ +	24	-	0	-	-
19	P 19	++	27	+++	4.03	-	+
20	P 20	++	21	+++	3.42	-	-
21	P 21	+	24.5	+++	3.64	-	-
22	P 22	-	0	++	2.42	-	-
23	P 23	-	0	-	0	-	-
24	P 24	-	0	-	0	-	-
25	P 25	+	14	+++	4.36	-	-
26	P 26	-	0	+++	3.75	-	-

Enzyme production: - = Nil; + = Present; ++ = Moderate; +++ = High

Selection of best isolates

Rhizobacterial isolates for further studies were selected based on the data of PGP traits and PAHs degradation in the screening studies. Rhizobacterial isolate, P 14 showed the best performance with reference to PAHs degradation and PGP traits, hence selected for identification and characterization.

Identification and Characterization

Colonies of P14 isolate are small, irregular, flat with an undulate margin and appeared shiny light cream in colour. Colonies were rough with dry texture, Gram positive, rod shaped, motile and sporulating. The isolate, P 14 on biochemical characterization, exhibited positive reactions to utilization of ornithine citrate, nitrate reduction, Voges-Proskauer's test, catalase, lipase and amylase enzyme tests. Similarly, it has utilized several carbohydrates for its growth as it showed positive reactions to carbohydrates like cellobiose, saccharose, trehalose, glucose and esculin.

Table 5
Production of different enzymes by rhizobacterial isolate P14

S. No.	Tests Performed	Isolate P 14
1	ONPG	-ve
2	Lysine Decarboxylase	-ve
3	Ornithine Utilization	+ve
4	Urease	-ve
5	Phenyl Alanin Deamination	-ve
6	Nitrate Reduction	+ve
7	H_2S Production	-ve
8	Citrate Utilization	+ve
9	Voges-Proskauer's Test	+ve
10	Methyl Red	-ve
11	Indole test	-ve
12	Malonate Utilization	+ve
13	Cytochrome Oxidase	-ve
14	Catalase	+ve
15	Lipase	+ve
16	Amylase	+ve

Table 6
Utilization of different carbohydrates by selected rhizobacterial isolate P14

Sl. No.	Test performed	Isolate P14
1	Esculin Hydrolysis	+ve
2	Arabinose	-ve
3	Xylose	-ve
4	Adonitol	- ve
5	Rhamnose	-ve
6	Cellobiose	+ve
7	Mellibiose	-ve
8	Saccharose	+ve
9	Raffinose	-ve
10	Trehalose	+ve
11	Glucose	+ve
12	Lactose	-ve

Upon molecular characterization selected bacterial isolate was identified as *Bacillus cereus* CPOU13 and its accession number is KJ626301.

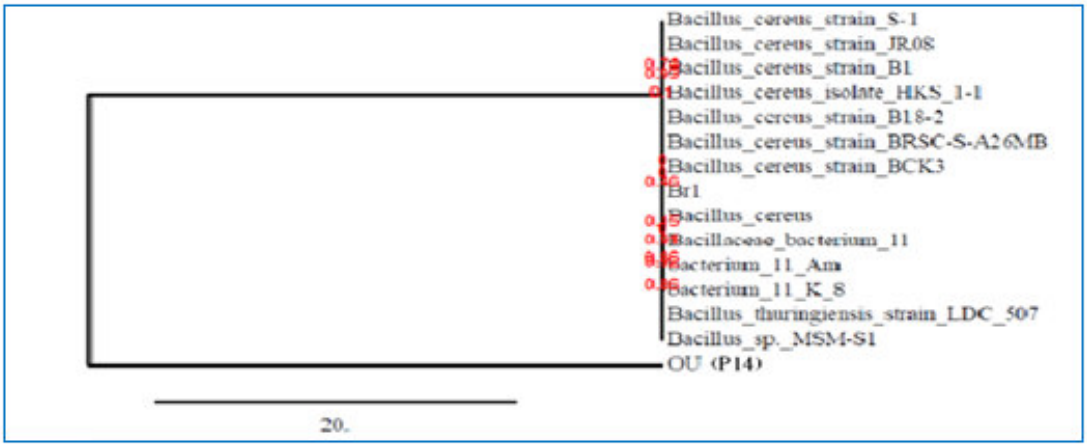

Figure 1
Phylogenetic relationship of P14 isolate

DISCUSSION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous, persistent organic pollutants that originate from natural and anthropogenic sources[19]. Crude oil based industries at different processing stages release high amounts of PAHs into the environment[20]. Wide array of soil microorganisms and in particular of rhizobacteria participates in degradation of PAHs[21,22]. Rhizosphere soils in PAHs contaminated sites accommodate huge number and high diversity of hydrocarbons degrading bacteria than uncontaminated soils[23]. Application of these rhizobacteria to PAHs contaminated soils improves amelioration of PAHs in non-native soils also[24]. In our study, a total of 26 PAHs degrading rhizobacteria were isolated from *Tridax procumbens*, *Cynodon dactylon* etc., belonging to Gram-positive and Gram-negative groups. The isolates were either bacilli or cocci. Mueller et al[21] suggested that in spite of the xenobiotic properties, a variety of genera of Gram-positive and Gram-negative bacteria, fungi and algae have been isolated and characterized for their ability to utilize PAHs. The isolates were assessed for their ability to adapt, utilize and degrade the polycyclic aromatic hydrocarbons like phenanthrene, anthracene and pyrene. Most of the strains isolated from natural PAHs contaminated sites responded positive to the PAHs test while few isolates failed to utilize them. Microorganisms in general and bacteria in particular have the ability to adapt to local environmental conditions and as a result of their exposure to many types of polycyclic hydrocarbons in nature, they might have acquired the quality of utilizing these contaminants even as a nutritional source[25]. *Mycobacterium austroafricanum* GTI-23 utilized phenanthrene, fluoranthene and pyrene as sole source of carbon and energy[26]. Dean-Ross et al[7] reported such a variation among the strains in utilizing phenanthrene, anthracene and pyrene. This may be attributed to the difference in catabolic potential expression in utilizing the PAHs[27] and the isolate P 14 showed maximum growth which may due to catabolic potential on PAHs enriched media. Plant growth promoting rhizobacteria (PGPR) are capable of promoting plant growth by secreting the products like ammonia, HCN, indole acetic acid, phosphatase, chitinase, cellulase, protease[28] etc.. PGPR have been studied for their ability to assist the uptake of nutrients from the environment and help preventing plant diseases. Many studies have been suggested PGPR strains are precious tools for effective bioremediation systems because of their broad range of ability to metabolize natural and xenobiotic compounds[29,30]. Nitrogen is a

macronutrient and main limiting factor for plant growth. It has considerable importance in nutrient cycling[31] and available to plants in the forms of $NH4^+$, NO^{-3} . In natural soil systems, high percentage of nitrogen is contributed by bacterial nitrogen fixation[32]. Nitrogenase enzyme complex of bacteria converts the molecular nitrogen to ammonia[33]. In this study, many isolates produced ammonia in peptone water and in that, 12 isolates, showed moderate to high production. These results are in conformity with the earlier reports of Kumar et al[34]; Rajithasri et al[35]. Hydrogen cyanide (HCN) is a secondary metabolite of many rhizobacteria that inhibit the growth of soil fungal pathogens[36,37]. Bacterial strains, *Alcaligenes, Aeromonas, Bacillus, Pseudomonas* and *Rhizobium* are known to produce HCN[38]. Seven of the 26 PAHs degrading isolates produced HCN moderately while 5 isolates produced low quantities. Production of indole acetic acid is a characteristic of many rhizobacteria that trigger many physiological reactions in plants[39]. A number of bacteria have the ability to produce indole acetic acid. They include *Azospirillum, Aeromonas, Azotobacter, Bacillus, Burkholdera*, *Enterobacte, Pseudomonas, Rhizobium* etc. However, the potential for indole acetic acid production varies among bacterial strains. Lwin et al[40] assessed production of indole acetic acid among 18 rhizobacteria isolated from Mandalay region, Myanmar and reported high production (121.1µg/ml) of indole acetic acid. *Pseudomonas aeruginosa* has been reported to produce indole acetic acid up to 80 µg/ml[41]. In the present study, the isolates exhibited great variation in producing indole acetic acid, 19 isolates out of 26 produced indole acetic acid in the range of 3.5 to 86µg/ml. Indole acetic acid production was high (86µg/ml) in isolate R1 and its lowest level 1.5µg/ml. Phosphatases of rhizobacterial origin produced growth enhancement effect in different plant species through phosphate solubilization[42]. Phosphatase production can be found among bacterial genera of *Bacillus, Burkholdera, Enterobacter, Klebsiella, Kluyvera, Pseudomonas*[43] etc. Phosphatase activity by the 26 rhizobacteria ranged from 11 to 55units/ml, and isolate P14 showed maximum phosphatase activity. The cell wall degrading enzymes, such as chitinase, cellulase, glucanase, protease etc. degrade fungal cell walls and play a key role in biocontrol of fungal pathogens[44]. These lytic enzymes hydrolyze a wide range of polymeric compounds, including chitin, proteins, cellulose, hemicellulose and interfere with growth and metabolism of pathogens[45]. Ruchi et al[46] isolated 26 protease producing rhizobacterial strains from apple and pear plants and reported that protease activity among the isolates was within the range of 1.6–5.5units/ml. In the present investigation, 18 isolates showed protease activity ranging from 1.24 to 5.32units/ml, isolate P14 showed maximum (5.32units/ml) activity. However, cellulase production was not detected among all the 26 isolates except for P 9. Similarly, chitinase activity was very less among the isolates and only P7, P14 and P19 exhibited the chitinase activity. Many PAHs degrading isolates in the present study have more than one PGP traits and produced PGP substances. However, variation among strains was observed in expressing PGP trait. The isolate, P 14 proved as most efficient PAHs degrading PGPR strains among the 26 bacteria. Classical approach to identify an unknown bacterium was proceeded with morphological, biochemical and molecular characterization. Morphologically, the isolate P 14 was Gram-positive, rod shaped, motile and sporulating bacterium. Biochemically the isolate P14 was positive to the tests for ornithin, citrate utilization, nitrate reduction, Voges Proskaure's, catalase, lipase and amylase. Molecular characterization based on 16S rDNA sequencing is a routine method[47]. Phylogenic dendrograms reflect the relation with other strains of bacteria. Comprehensive analysis of nucleotide bases of bacterial 16S rDNA with appropriate bioinformatics tools allows identification of unknown bacteria[48]. Based on morphological, biochemical and molecular characterization the isolate P14 was identified and named as *B. cereus* CPOU13. Species of *Bacillus* are the major ones of common hydrocarbon degrading microorganisms found in many types of

contaminated sites. Shimura et al[49] isolated a strain of *Bacillus*, *Bacillus* sp. JF8 that possesses the capability to degrade naphthalene. Yuliani et al[50] isolated 4 strains of *Bacillus* those can degrade pyrene and phenanthrene. Calvo et al[51] studied degradation of naphthalene by a strain of *Bacillus pumilus* isolated from oil sludge. Many studies reported the strains of *B. cereus* are potent PAHs degrader[52]. Simultaneously many researchers reported, *Bacillus* strains as excellent producers of plant growth promoting traits[53]. In the present study among the isolated 26 PAHs degrading rhizobacteria only one isolate P14 or *B. cereus* CPOU13 is found to be amenable for further research like rhizodegradation of PAHs along with plant growth promotion.

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