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Effective Polycyclic Aromatic Hydrocarbons Degrading Rhizobacteria with PGP Traits from Plants Growing on Fly Ash in the Premises of Thermal Power Station–Kothagudem, Telangana State, India

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Abstract: ***Polycyclic aromatic hydrocarbons (PAHs) degrading rhizobacteria with plant growth promoting (PGP) traits play key roles in rhizoremediation systems. Their isolation from various PAHs contaminated sites is amenable for effective PAHs remediation. In our study, we have isolated 6 (six) PAHs degrading rhizobacteria from the plants growing on fly ash released from thermal power station (TPS) of Kothagudem located in Paloncha, Khammam district, Telangana state, India. The isolates were screened for the degradation of three test PAHs such as phenanthrene, anthracene and pyrene and identified that the isolates have the capabilities to degrade more than one type of PAHs in minimal salt medium (MSM) when enriched with three PAHs. The isolates again screened for their abilities to produce PGP traits such as ammonia, HCN, IAA and the enzymes like phosphatase, protease, chitinase and cellulase. Among the screened six isolates the isolate R1, produced good qualities of PAHs degradation and PGP traits. Thus isolate R1 was further assayed for characterization and identification. The characterization was proceeded with morphological, biochemical and molecular methods. The compilation of characterization data helped us to identify the isolate as Bacillus subtilis SPC14.***

Keywords: Polycyclic aromatic hydrocarbons, plant growth promoting traits, thermal power station, phenanthrene, anthracene, pyrene

1. Introduction

Energy is the nucleus for the development of every nation across the world. In developing countries energy requirement is very high to meet rapid growth, development of industries and their productivity. During electricity generation processes coal carrying units at thermal power stations (TPS) release deleterious compounds like PAHs. PAHs are the main resultants of incomplete combustion (Seuss et al. 1976). Thermal power stations release fly ash that heavily included with PAHs, disperse into surroundings and contaminate the atmosphere, water and land ecosystems (Arditsoglou et al. 2004). Sometimes contamination of PAHs may reach distant places due to its low weight and easy dispersion (Donahue et al. 2006).

Polycyclic aromatic hydrocarbons are a kind of organic pollutants that are highly susceptible carcinogens and affect the human health in several ways (Wenzl et al. 2006). This is the subject and enforced move of scientific world to emergent amelioration of PAHs from land ecosystems. The recent research has been much focused the importance of rhizosphere inhabited PAHs degraders from rhizosphere soils (Mohan et al. 2006).

PAHs degrading rhizobacteria with PGP traits or having ability to produce ammonia, HCN (hydrogen cyanide), IAA (Indole acetic acid) and extracellular enzymes like phophatase, protease, chitinase, cellulase confers additional benefits. These traits help to promote plant growth and degradation of PAHs which depends on plant health and cooperation (Reed et al. 2005). The present research is mainly aimed at the isolation and identification of PAHs degrading PGP traits possessing rhizobacteria from the plants growing on fly ash located at Kothagudem Thermal Power Station located in Paloncha, district of Khammam, Telangana State, India.

2. Materials and Methods

2.1. Isolation of Polycyclic Aromatic Hydrocarbons (PAHs) degrading rhizobacteria

Rhizosphere soils of *Tridax procumbens*, *Ocimum sanctum* and *Cleome gynandra* plants growing on fly ash were collected as described by Bashan et al. (1993). The method of enrichment and isolation of PAHs degrading bacteria was adopted from Dean-Ross et al. (2001). Isolates were purified according to the procedure of Kiyohara et al. (1982). The ability of the isolate to utilize PAHs was tested using three test PAHs such as phenanthrene, anthracene and pyrene according to the method of John et al. (2012).

2.2. 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 along with HCN and extracellular enzymes. Production of ammonia was assessed as described by Cappuccino and Sherman, (1992). Production of auxin was assessed by the procedure of Gordon and Paleg, (1957). HCN was determined by following the method of Bakker and Schipper, (1987) and

chitinases was screened by the method of Rodriguez-Kabana (1983). Production of phosphatases by observing phosphate solubilization (Goldstein, 1986). Quantification of phosphatase activity was done by adopting the method of Banerjee et al. (2012). Production of proteases was screened on skim milk agar medium (Denizci et al. 2004) and the production of cellulase enzymes on M9 medium amended with carboxy methyl cellulose.

2.3. Characterization of selected isolates

Morphological characteristics of selected bacterial colonies such as 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. (1995). Phylogenetic tree was constructed for selected isolate by retrieving the bacterial strain names having highest scores of percentage similarity and Neighbor-joining method (Saitou and Nei, 1987). The 16S rDNA sequences were submitted to NCBI GenBank by MyBaklt tool and got the accession number.

3. Results

3.1. Isolation of PAHs degrading rhizobacteria

Six Rhizobacteria isolates having the ability to degrade PAHs were isolated on MSM enriched with a test PAHs phenanthrene (100ppm). The names given to the 6 rhizobacterial isolates were R1, R2, R3, R4, R5, R6 and their characteristics are presented in Table 1.

Table 1: Names and characterstics of rhizobacteria isolated from fly ash at KTPS

Isolate	Morphology
R 1	Gram positive, *Bacillus*
R 2	Gram positive, *Coccus*
R 3	Gram positive, *Coccus*
R 4	Gram positive, *Coccus*
R 5	Gram positive, *Bacillus*
R 6	Gram positive, *Coccus*

All the isolates were screened for their ability to grow on MSM enriched with any one of three test PAHs such as phenanthrene, anthracene or pyrene (100ppm) as sole source of carbon and growth was recorded in terms of optical density (OD) at 600nm and the results are presented in Table2. Among the six isolates screened for degradation of three test PAHs the isolate R1 showed good growth on three test PAHs and isolates R2 grew best on anthracene.

Table 2: Screening of rhizobacterial isolates for the degradation of phenanthrene, anthracene or pyrene

Sl. No.	**Isolate**	**Phenanthrene**	**Anthracene**	**Pyrene**
1	Control (Without PAHs)	0	0	0
2	R 1	0.41	0.38	0.21
3	R 2	0.01	0.32	0.01
4	R 3	0.003	0.02	0.01
5	R 4	0.004	0.03	0.001
6	R 5	0.002	0.04	0
7	R 6	0.003	0.04	0.01

3.2. Screening of PAHs degrading isolates for PGP traits

All the six isolates produced ammonia from low to moderate levels. Among the six isolates, two isolates(R1, R4) exhibited the production of HCN and isolate R1 showed moderate production. In connection to IAA two isolates produced IAA and R1 isolate produced maximum of 42µg/ml.

Table 3: Production of Ammonia, IAA and HCN by the six isolates

S.No.	Isolate	Ammonia Production	IAA (µg/ml)	HCN Production
1	R 1	+++	42	++
2	R 2	+	3.5	-
3	R 3	+	0	-
4	R 4	+	1.5	+
5	R 5	++	0	-
6	R 6	++	7	-

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

Production of extracellular enzymes such as phosphatase, protease, cellulase and chitinases by rhizobacteria is of great importance in promoting plant growth and biocontrol. In view of this, all the isolates were tested for extracellular enzyme production and the results are presented in Table 4. Phosphatase production was reported in isolate R1 (45units/ml) only. High protease activity (5.03units/ml) was found in isolate R1 and it was followed by remaining 5 isolates. Very low production of chitinases was exhibited by the isolate R1 and other isolates were failed to produce chitinases at all. No rhizobacterial isolate produced the enzyme cellulase.

Table 4: Production of extracellular enzymes

Sl. No.	Isolate	Phosphatase		Protease		Cellulase	Chitinase
		Qualitative Test	Quantity (Units/ml)	Qualitative Test	Quantity (Units/ml)	Qualitative Test	Qualitative Test
1	R 1	+++	45	+++	5.03	-	+
2	R 2	-	0	+	1.34	-	-
3	R 3	-	0	+	1.83	-	-
4	R 4	-	0	+	1.79	-	-
5	R 5	-	0	+	1.42	-	-
6	R 6	-	0	+	1.67	-	-

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

3.3. Selection and characterization of best PAHs degrading PGPR isolate

The promising isolates were selected for further studies on the basis of PGP traits and PAHs degradation after the screening tests. Only one isolate R1 showed the best abilities to PAHs degradation and PGP traits. Hence it was selected for characterization and identification.

Morphologically, the colonies of isolate R1 are circular, large, smooth, flat with undulate margins and appeared light cream in colour. The isolate was Gram positive, rod shaped, motile and sporulating (Fig. 1)**.** The isolate, R1 on biochemical characterization exhibited positive reactions to lysine decarboxylase, ornithine utilization, urease, citrate utilization, VogesProskauer's, catalase, lipase and amylase enzymes production tests and utilized carbohydrates like cellobiose, saccharose, trehalose, glucose and esculin (Fig. 2). In molecular characterization using 16S rDNA, the isolate exhibited maximum identity (99%) with several strains of *Bacillus subtilis* on NCBI microbial blast when query cover was 99%. Phylogenetic tree was constructed depending on the highest similarities showing bacterial sequences using Neighbour joining method (Fig. 3). The 16S rDNA sequence of the isolate was submitted online to NCBI Genbank, got the accession no. KM077281 and named it as *Bacillus subtilis* SPC14.

4. Discussion

Coal dependant TPS combust huge quantities of coal and release fly ash. Fly ash is a likely source for carcinogenic PAHs and heavily pollutes surroundings (Donahue et al. 2006).This leads to the development of microbial communities that can tolerate the effects of PAHs or have the abilty to degrade PAHs. Special ecological niches such as 'rhizosphere' of plants with high microbial activity is primarily considered as highly efficient region for the removal of PAHs and frequently preferred to isolate PAHs degrading bacterial strains (Yu et al. 2003). In rhizosphere degradation of PAHs is sophistically coordinated by bacterial population and their interactions (Divya and Kumar, 2011).

Bacteria maintain adaptability to local contaminants and nutritional sources (Siciliano et al. 2003). Several strains of bacteria possess ability to utilize more than one type of PAHs for their growth and metabolism (Hunter et al. 2005; Pizzul et al. 2007). For the instance, *Mycobacterium austroafricanum* GTI-23 can utilized phenanthrene, fluoranthene and pyrene as sole source of carbon and energy (Bogan et al. 2003). Bisht et al. (2010) isolated some strains of bacteria utilizing anthracene and naphthalene from the rhizosphere of *Populus deltoides*. In the present study, we have isolated six PAHs degrading rhizobacteria from *Tridax procumbens*, *Ocimum sanctum*, *Cleome gynandra* growing on fly ash contaminated site and all the isolates have the ability to degrade more than one of PAHs for their growth.

Rhizobacteria are indispensable part of rhizosphere and can secrete many valuable products to promote the plant growth in many adverse conditions and show a broad range of ability to metabolize organic pollutants like PAHs (Pattern and Glick, 1996; Siciliano and Germida, 1998; Glick, 2001). Effective degradation of PAHs can be achieved by incorporating PAHs degrading bacteria with PGP traits into remediation systems. In our study, only two isolates produced HCN. Production of IAA is a common feature of many rhizobacteria that trigger many physiological reactions in plants. Lwin et al. (2012) assessed the production of IAA among 18 rhizobacteria isolated from Mandalay region, Myanmar and reported high production (121.1µg/ml) of IAA. In the present study 4 isolates produced IAA in the range of 1.5 to 42µg/ml and production of IAA was very high in isolate R1.

Rhizobacteria have ability to produce phosphatase that provides an advent of plant growth. SundaraRao and Sinha, (1963) reported that *Bacillus* strains from wheat rhizosphere solubilized 112-157mg/L of phosphate in 14days of incubation. In our study, phosphatase activity was observed in isolate R1 only. Ruchi et al. (2012) 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, all the isolates showed protease activity ranging from 1.34 to 5.03units/ml. However, cellulase production was not detected among all the 6 isolates. Similarly, chitinase activity was very less among the isolates and found in isolate R1only. Many isolates of our study exhibited multi-PGP traits.

In the screening for both PAHs degradation and PGP traits most of the isolates showed both qualities. However variation among strains was observed in expressing PAHs degradation and PGP traits. Basing upon the collective information of PAHs degradation and PGP traits we selected isolate R1 for further studies.

Traditionally, identification of an unknown bacterium is done with morphological, biochemical and molecular characterization. Morphologically, the isolate R1 was Gram-positive, rod shaped, motile and sporulating bacteria. Biochemically the strain was positive to enzyme tests of ornithine utilization, nitrate reduction, citrate utilization, VogesProskaure's, catalase, lipase and amylase. Molecular characterization based on 16S rDNA sequencing is a routine method for bacterial identification and has been used in all disciplines of microbiology (Lane et al. 1985). Comprehensive analysis of nucleotide bases of bacterial 16S rDNA with appropriate bioinformatics tools allows identification of unknown bacteria (Ludwig et al. 1998). Based on morphological, biochemical and molecular characterization, the isolate R1 has been identified as *B. subtilis* SPC14.

5. Future Scope

Knowledge regarding the bacterial biodegradation of PAHs has advanced in the last decade. A number of PAH-degrading strains have been isolated and characterized. PGPR present an alternative to the use of chemicals for plant growth enhancement in many different applications. Extensive research has demonstrated that PGPRs could have an important role in PAHs degradation, PGP traits, enzymatic activity and which might be useful to biodegrade

sorbed PAHs in soils and sludge. Among the isolated strains R1 with better degradation was identified based on gene sequence of 16SrDNA, named it as *Bacillus subtilis* SPC14 and got the accession no. KM077281. Our present results are in conformity with many other researchers. This strain has shown degradation over a broad range of PAHs in turbidity studies. The present study indicates that the isolated strain *Bacillus subtilis* SPC14 would prove to be a promising candidate for bioremediation of PAHs contaminated sites.

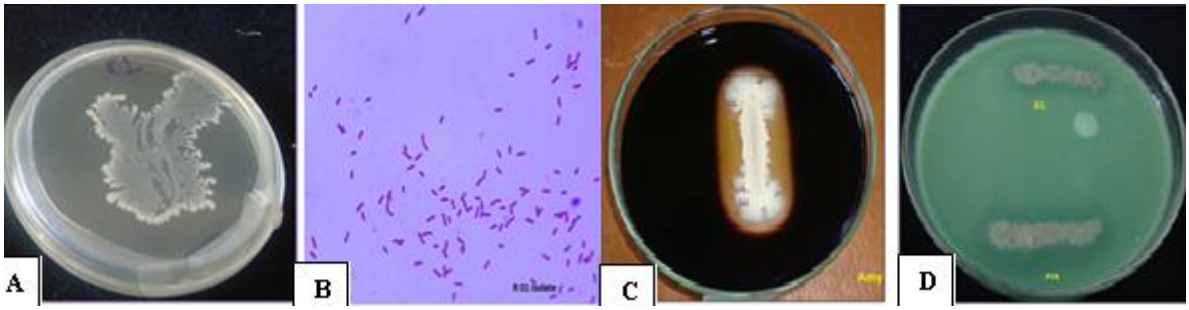

Figure1: Isolate R1: A. colony morphology. B. Gram staining, C. Amylase activity, D. Lipase activity

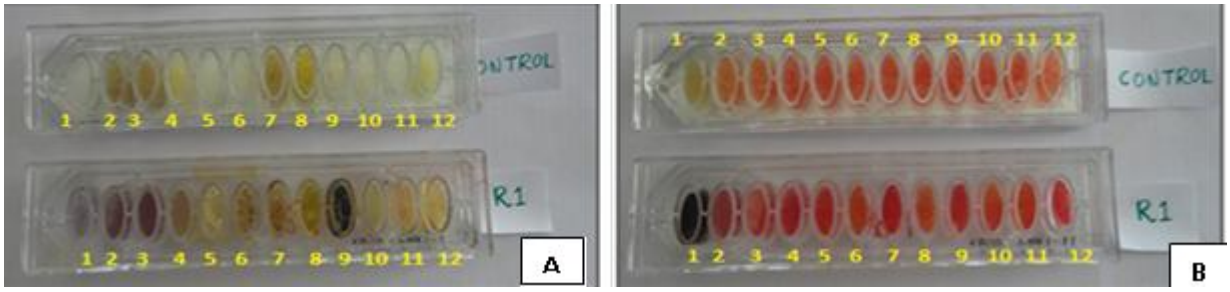

Figure 2: Biochemical characterization of isolate R1 (A) Production of different enzymes (B) Utilization of carbohydrates

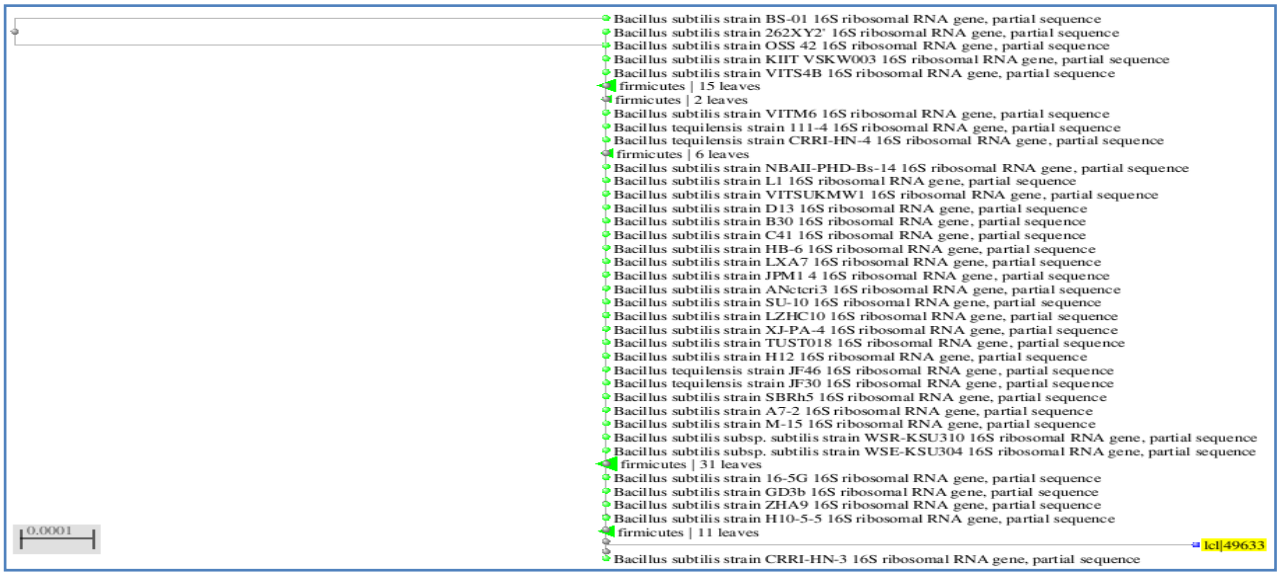

Figure 3: Phylogenetic relationship of the isolate R1 with other strains of *Bacillus subtilis* SPC14

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