INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY, BIOLOGY AND CHEMISTRY

Research Article

Isolation of polycyclic aromatic hydrocarbons (PAHs) degrading bacteria from an open coalmine samples- Kothagudem, Telangana, India

M. Poornachander Rao

Government Degree College, Mahabubabad, Waranagal (Dist.), Telangana, India- 506101.

ABSTRACT

The present investigation conducted to isolate polycyclic aromatic hydrocarbons (PAHs) degrading bacteria strains from coal samples of an opencast coalmine located in Kothagudem of Khammam district, Telangana state, India. A total of five (5) bacterial strains were isolated from this coalmine using minimal salt medium (MSM) that priory enriched with phenanthrene, a PAHs compound and observing lyses zone formations on Petriplates contained media. Primary microscopic observations of the bacterial isolates revealed that three isolates are Gram-positive and two isolates are Gram-negative bacteria and all are rod shaped. The isolates were further screened for their ability to grow on three test PAHs compounds such as phenanthrene, anthracene and pyrene provided as sole source of carbon in the media. In the study, all the isolates recorded good ability to grow on these three test PAHs. The isolates under morphological characterization are significantly differed. Biochemical characterization was done by using 'Rapid bacterial identification kits (Himedia) and production abilities of different enzymes on special media by the isolates were determined. All the strains reacted differently with the reagents of biochemically characterizing media. Finally the isolates are characterized at molecular level by sequencing 16S rDNA genes. The bacterial strains finally indentified as *Bacillus cereus* KMM6, *Bacillus pseudomycoides* KMM7, *Pseudomonas aeruginosa* KMM8, *Bacillus cereus* KMM9 and *Pseudomonas stutzeri* KMM10.

Key Words: PAHs, Minimal salt medium, Phenanthrene and Characterization.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) of coal origin have great interest in environmental research since huge state of coal mining for energy production around the world. Our nation, India stands at 3rd position among the top eleven hard coal producing countries with 0.4 billion tons after China and USA. Worldwide hard coal production has increased from 1 to 4.96 billion tons from 1900 to 2005. In 2004, China, the U.S.A. and India produced about 2, 1 and 0.4 billion tons of hard coal and held about 60, 100 and more than 80 billion tons of reserves respectively. These activities around the globe are effecting the present global environment and its inhabitants[1].

Bacteria are ubiquitous in existence and reported from all environments segments that ranged from natural to extreme[2]. They adapt too many adverse conditions and exhibit high tolerance levels. Earlier researchers registered the existence of bacteria in extreme high and low temperatures, high pressure, acidified habitats etc. However existence of high density of bacteria restricted to the places like coalmines and till dated few bacterial strains reported in coal beds and their particles from coal mines[3]. This situation is mainly due to unfavorable growth conditions in those sites for the existence of natural and commonly found soil microorganisms[4]. Coalmine regions are prevalent with adverse bacterial growth conditions like high temperatures, low pH and nutrients deficiency[5]. However, scanty studies reported indigenous group of bacterial species from natural coal samples[6, 7]. Biodegradation of PAHs using indigenous bacterial strains from PAHs polluted sites like coalmines has getting prime

importance in recent research trends because of their many advantages like harmless, eco-friendly and unchanged medium quality where it operated. This kind of research makes successive development of potent PAHs degrading bacterial communities or their consortia that more compatible for any PAHs contaminated sites. For this reasons, the present investigation conducted to isolate PAHs degrading bacteria strains from coal samples of an opencast coalmine in Kothagudem of Khammam district, Telangana state, India.

MATERIALS AND METHODS

Isolation of PAHS degrading bacteria:

Coal samples were collected aseptically with the help of spatulas from opencast coalmines located in Khammam district and aseptically transported to the laboratory. The method of enrichment and isolation of PAHs degrading bacteria is done by using minimal salt medium (MSM)[8]. Samples added into MSM enriched with phenanthrene that priory sterilized by autoclaving at 121º C for 15 min. 0.2 ml of acetone solution included with required amount of phenanthrene (100 ppm) added under aseptic conditions to the MSM. Culture flasks added with pre-serially diluted coal samples and incubated on an orbital shaker at a speed of 150 rpm for 7 days. After the completion of incubation, 1 ml of culture added to fresh MSM for better enrichment. After four transfers, the enrichment culture poured onto the MSM agar Petriplates that priory sprayed with test PAHs compounds (phenanthrene, anthracene and pyrene) separately. PAHs degrading isolates were identified by the formation of clear zones around the bacterial colonies which indicates PAHs utilization.

Purification of the PAHS degrading bacteria:

Cultures of PAHs degrading bacteria were purified by repeated streaking method[9]. This was made by repeated streaking of single colonies on nutrient agar medium (NAM) and subsequent transfers to PAHs sprayed MSM Petriplates. Purity of the isolates was confirmed by microscopy method. Pure cultures grown in Luria Bertani (LB) broth medium then suspended in 15% glycerol and stored at -80º C in specialized refrigerators until the use for further studies.

Screening the isolates for the degradation of test PAHS:

Growth ability of PAHs degrading bacteria on test PAHs (phenanthrene, anthracene or pyrene) was tested by growing each isolate in separate large test tubes containing 25 ml of screening medium (MSM) supplemented with 100 ppm concentration of phenanthrene, anthracene or pyrene which were pre-dissolved in acetone and added to each tube after autoclaving. In control samples no test PAHs was added. Thereafter, the test tubes incubated at room temperature (28 ± 2º C) and shaken at 130 rpm speed for three days. The PAHs degradation ability of each isolate by utilizing phenanthrene, anthracene and pyrene results in increase of medium turbidity. Growth was measured in terms of optical density (OD) at 600 nm using a UV spectrophotometer[10].

Characterization and Identification of PAHS degrading bacterial isolates morphological characterization:

Morphological characteristics of the bacterial isolates were studied using microscopy methods. Colony characteristics like shape, size, elevation, surface, margin, colour, pigmentation and individual bacterium characteristics like shape, motility, spore formation, reaction with Gram stain were recorded as per standard microbiology methods and protocols.

Biochemical characterization of PAHS degrading bacterial isolates:

Biochemical characteristics like the production of different enzymes in special media were determined by using 'Biochemical Characterization Kit' KB003 Hi 25 of Himedia. Protocol, instructions and results analysis followed according to the manufacturer instructions. Biochemical characterization and their application in bacterial taxonomy are conducted in this study[11]. The catalase test also performed[12]. For the screening of lipase production, cultures of isolates streaked on NAM and incubated for 24 h under standard growth conditions. After the incubation, saturated solution of $CuSO_4$ poured onto the growing cultures. Formation of clear zones indicated the production of lipases. Amylase production identified by growing the isolates on NAM amended with starch containing agar plates and incubated for 24 h. After the incubation, iodine solution poured on to the culture. Formation of clear zones around the colonies indicated the production of amylase.

Molecular characterization of PAHs degrading bacterial isolates:

Molecular characterization for PAHs degrading bacteria is done by using 16S rDNA gene sequences[13]. DNA extraction method was adopted from Gauthier[14]. Amplification of 16S rDNA region was carried out in a polymerase chain reaction (PCR) machine (PCR Minicycler, Model PTC 80, MJ Research, USA) by using universal primers fD 1 (5' GAGTTTGATCCTGGCTCA 3') and rP 2 (5' ACGGCTACCTTGACTT 3'). Nucleotide bases

were sequenced using a 'DNA Sequencer' and analyzed by bioinformatics tool, Basic Local Alignment Search Tool (BLAST) available at National Center for Biotechnology Information (NCBI) website. The 16S rDNA sequences were further compared to available reference nucleotide sequences from the database of NCBI and European Molecular Biology Laboratory (EMBL). Similarity percentages between known and isolate's unknown sequences were obtained after BLAST analysis. Phylogenetic trees were constructed for each isolate by retrieving the reference bacterial strains names having highest scores of percentage similarity using Neighbor-joining method. Finally, the 16S rDNA sequences submitted and deposited in NCBI GenBank to get the accession numbers.

RESULTS

Isolation of polycyclic aromatic hydrocarbons (PAHs) degrading bacteria from coal samples:

Polycyclic aromatic hydrocarbons (PAHs) degrading bacteria were isolated from coal samples of opencast coalmines located in Khammam district of Telangana State, India. These sites fully covered with coal and proposed to be containing PAHs. These isolates are designated as 'KMM' series. In the present study, 5 PAHs degrading bacteria were isolated from coal samples on minimal salt medium (MSM) broth enriched with a PAH compound phenanthrene at 100 ppm concentration (Table 1). After the isolation, they were purified by repeated streaking and sub-culturing on MSM agar and nutrient agar medium (sprayed with phenanthrene).

Screening the bacterial isolates for phenanthrene, anthracene and pyrene degradation:

All PAHs degrading isolates were screened for their ability to grow on MSM enriched with phenanthrene, anthracene or pyrene at 100 ppm concentration as sole source of carbon and growth was recorded. The results are presented in Table 2.

All the isolates utilized phenanthrene, anthracene and pyrene for their growth and metabolism. Growth recordings of the isolates are good at 600 nm. This experiment results suggested that all the bacterial isolates have the good ability to utilize three tests PAH compounds (phenanthrene, anthracene and pyrene) as a source of carbon.

Characterization of the PAHs degrading bacterial isolates

After the conduction of screening tests for PAHs degradation characterization and identification of the isolates was done depending upon morphological, biochemical and molecular characteristics data.

Morphological characterization:

All the cultures of PAHs degrading bacterial isolates were examined for colony and cell morphologies, Gram reaction and spore formations. Colony morphologies of all the isolates were examined by growing isolates on nutrient agar. Colony morphological characteristics such as form, texture, color, margin, elevation and opacity of the isolates were studied and the results are presented in Table 3.

Biochemical characterization of the PAHs degrading isolates:

Biochemical characterization based upon the production abilities of different enzymes on special media was done using Himedia biochemical characterization Kit (KB003 Hi 25).

The isolate, KMM6 on biochemical characterization recorded positive reactions to the assays of Ornithine utilization, nitrate reduction, citrate utilization, Voges-Proskauer's, malonate utilization, catalase, lipase and amylase. On the other hand, the strain recorded negative reactions to the assays of ONPG, lysine decarboxylase, urease, phenyl alanin deamination, H_2S production, methyl red, Indole and cytochrome oxidase.

The isolate, KMM7 on biochemical characterization exhibited positive reactions to the tests of lysine decarboxylase, nitrate reduction, citrate utilization, Voges-Proskauer's, malonate utilization, catalase, lipase and amylase. On the other hand, the isolate exhibited negative reactions to the tests of ONPG, Ornithine utilization, urease, phenyl alanin deamination, H_2S production, methyl red, Indole and cytochrome oxidase.

The isolate, KMM8 on biochemical characterization using Himedia kit exhibited positive reactions to the tests of lysine decarboxylase, nitrate reduction, citrate utilization, malonate utilization, cytochrome oxidase, catalase, and lipase. On the other hand, the same strain exhibited negative reactions to the tests of ONPG, Ornithine utilization, urease, phenyl alanin deamination, H_2S production, Voges-Proskauer's, methyl red, Indole and amylase.

The isolate, KMM9 on biochemical characterization performed positive reactions to the tests of Ornithine utilization, nitrate reduction, citrate utilization, Voges-Proskauer's, malonate utilization, catalase, lipase and amylase. On the other hand, the strain performed negative reactions to the tests of ONPG, lysine decarboxylase, urease, phenyl alanin deamination, H_2S production, methyl red, Indole and cytochrome oxidase.

The isolate, KMM10 on biochemical characterization showed positive reactions to the tests of nitrate reduction, H_2S production, citrate utilization,

cytochrome oxidase, catalase and amylase. On the other hand, the strain showed negative reactions to the tests of ONPG, lysine decarboxylase, Ornithine utilization, urease, phenyl alanin deamination, Voges-Proskauer's, methyl red, Indole, malonate utilization and lipase.

Molecular characterization of the PAHs degrading isolates:

The strain, KMM6 upon molecular characterization with the aid of 16S rDNA gene sequencing method recorded the maximum identity (99%) and query cover of 100% with the strain, *Bacillus cereus* OU13 strain (KM26301.1). Phylogenetic tree for the isolate was constructed based on the highest gene similarity alignments (Fig. 1).

The isolate, KMM7 exhibited the maximum identity (99%) and query cover of 100% with *Bacillus pseudomycoides* strain BS6 16S ribosomal RNA sequences (Sequence ID: KU061612.1) (Fig. 2). The strain, KMM8 recorded the maximum identity (99%) and query cover of 100% with strain, *Pseudomonas aeruginosa* PAO1 (NC 002516.2) (Fig. 3). The strain, KMM9 recorded the maximum identity (99%) and query cover (100%) with many *Bacillus cereus* strains (Fig. 4). The isolate, KMM10 registered the maximum identity (99%) and query cover of 100% with *Pseudomonas stutzeri* strain A1501 (Sequence ID: NC009434.1) (Fig. 5).

DISCUSSION

Amelioration of the PAHs compounds from the environment is an important and enforced task due to its presence over the all environmental segments and their deleterious effect. Few bacterial strains tolerate toxic effects of PAHs and degrade them successfully in short durations. Biodegradation with the aid of such PAHs degrading bacterial strains to clean up PAHs contaminated soils and water bodies is more promising than physical and chemical methods that generally preferred for other types of pollutants[15]. These PAHs degrading bacteria acquire genetic adaptabilities to utilize PAHs compounds for their growth and metabolic activities after long term exposure to these pollutants and become indigenous strains[16]. In this concern coal, coal dust and coal products encourage the development of PAHs degrading abilities in bacterial strains as coal considered to the richest source for many PAHs[17, 18].

Identification of bacterial diversity that having the ability to degrade PAHs from coal samples is undertaken in the present study and five efficiently PAHs degrading bacterial strains isolated from coal samples of Kothagudem opencast coalmines. These bacterial strains were isolated from an opencast coalmine in Kothagudem on MSM priory enriched with phenanthrene, a PAHs compound and observing lytic zone formations on Petriplates containing media. Thereafter, continuous streaking of the isolate cultures on MSM and NAM and purified bacterial isolates. Finally the purified cultures are coded as KMM series. Primary microscopic observations of the bacterial isolates revealed that 3 isolates are Gram-positive and 2 isolates are Gram-negative bacteria and all are rod shaped. Similar results are published in earlier studies[19, 20, 21, 22].

Exposure to PAHs is never to single PAHs. Understanding what differences may occur in mixtures of PAHs gives an accurate assessment of the dangers of PAHs. Understanding the dynamics of single metabolism of PAHs and possible toxic effects is a necessary and this will guide to understand the accurately impact of PAHs and will guide to a well remediation strategies[23]. The isolates were further screened for their ability to grow on three test PAHs compounds such as phenanthrene, anthracene and pyrene provided as sole source of carbon in the media. All the isolates recorded good ability to grow on three test PAHs (phenanthrene, anthracene and pyrene). The results are coinciding with many earlier findings[24-26]. Based on their ability to degrade three PAHs all the isolates were selected for the characterization, identification and nomenclature.

Characterization and identification of PAHs degrading bacterial isolates proceeded with three stages *viz*., morphological characterization, biochemical characterization and molecular characterization. Further it was followed by compilation of this data to identify the strains. Morphological characterization was done by observing the isolates colonies and individual cell structures using microscopy methods. All the isolates are characteristically differed in microscopic observation. Biochemical characterization plays very important role in bacterial identification and this was done using 'Rapid bacterial identification kits (Himedia). In this, production abilities of different enzymes on special media by the isolates were determined. All the strains reacted differently with the reagents in media. Finally the isolates are characterized at molecular level using 16S rDNA sequences[27]. Molecular method that was used for the identification of bacteria is based on the analysis of the 16S rDNA gene and sequences of 16S rDNA are characterized by highly conserved regions, which help in the analysis. They also have regions of high variability, making it possible to define the evolutionary distance of given organisms[28]. 16S rDNA of the isolates were sequenced using universal primers, fD1 and rP2 and analyzed them with the

help of Basic Local Alignment Search Tool (BLAST) analysis. The results were compared with available reference nucleotide sequences from the database sequences deposited in NCBI and EMBL Genbanks. Based on gene similarity percentages of the isolates (16S rDNA gene sequences with other bacterial sequences) phylogenic trees were constructed using Neighbor-joining method and taxonomical positions of the isolates were determined.

Table 1
PAHs degrading bacterial isolates from coal samples of Khammam district

S. No.	Isolate code	Characteristics
1	KMM6	Gram- positive, rod shaped
2	KMM7	Gram-negative, rod shaped
3	KMM8	Gram-negative, rod shaped
4	KMM9	Gram- positive, rod shaped
5	KMM10	Gram- positive, rod shaped

Table 2
Growth of the isolates on MSM broth enriched separately with phenanthrene, anthracene or pyrene (100 ppm concentration)

S. No.	Code of the Isolate	Optical Density at 600 nm		
		Phenanthrene	Anthracene	Pyrene
1	Control (Without PAHs)	0	0	0
2	KMM6	0.04	0.09	0.08
3	KMM7	0.05	0.03	0.05
4	KMM8	0.05	0.04	0.03
5	KMM9	0.06	0.11	0.07
6	KMM10	0.04	0.03	0.05

Table 3
Morphological characterization of the PAHs degrading isolates

S. No.	Code of the Isolate	Characteristics
1	**KMM6**	Colonies are small, irregular, flat with undulate margin, have rough and dry texture and shiny light cream in colour and cells are motile and sporulating.
2	**KMM7**	Colonies are opaque, have rhizoid like bodies and white to cream in colour. Cells are non-motile and sporulating.
3	**KMM8**	Colonies are large, oval, convex, rough, white to cream in colour and also appearing bluish green. Cells are motile and non-spore forming.
4	**KMM9**	Colonies are small, irregular, flat with an undulate margin, have rough and dry texture and shiny light cream in colour. Cells are motile and sporulating.
5	**KMM10**	Colonies are smooth to rough, forming pits on the medium and white to off-white in colour. Rods of the Isolate are straight and slightly curved. Cells are motile and non-spore forming.

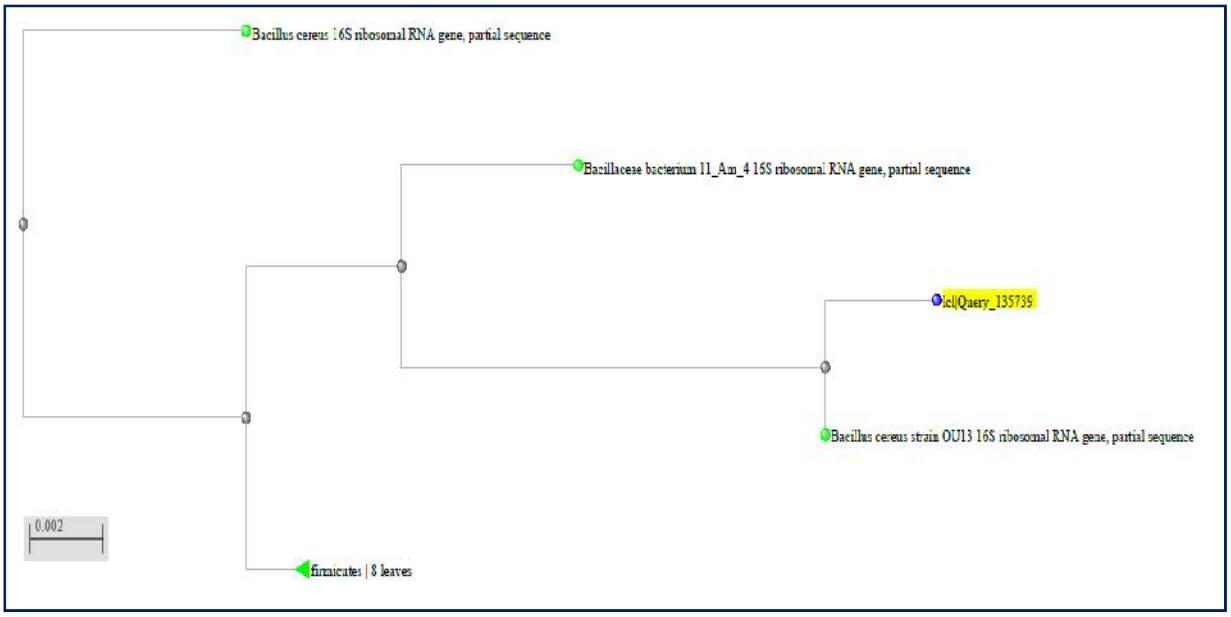

Fig. 1
Phylogenetic relationship of the isolate KMM6

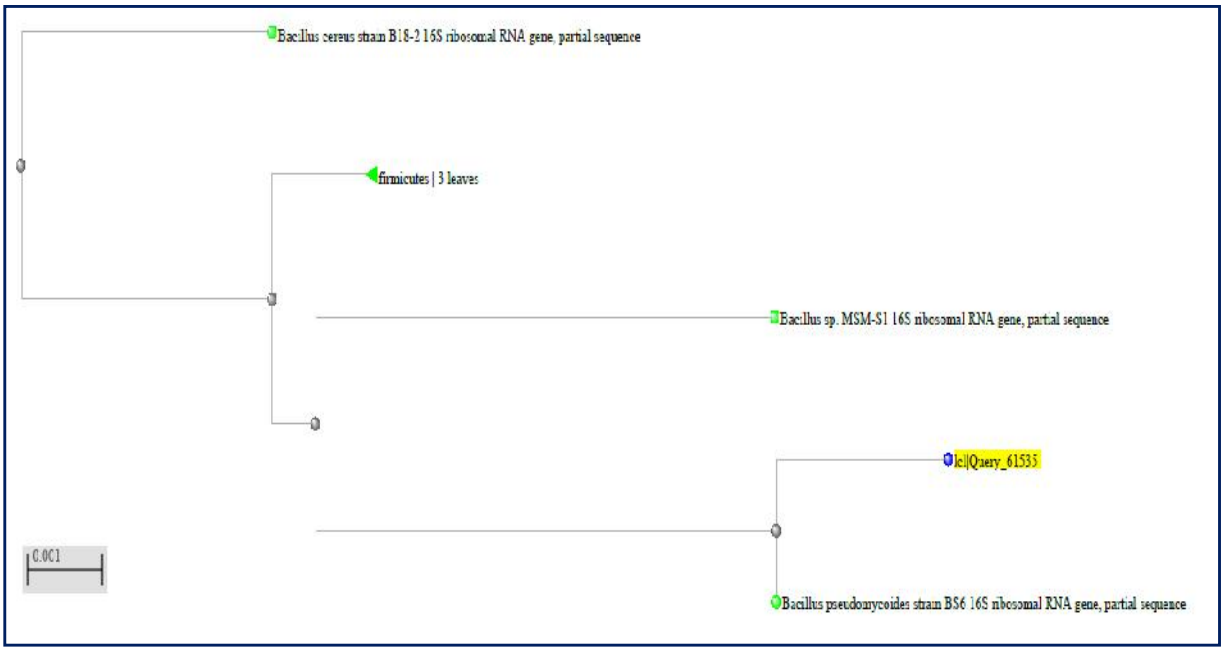

Fig. 2
Phylogenetic relationship of the isolate KMM7

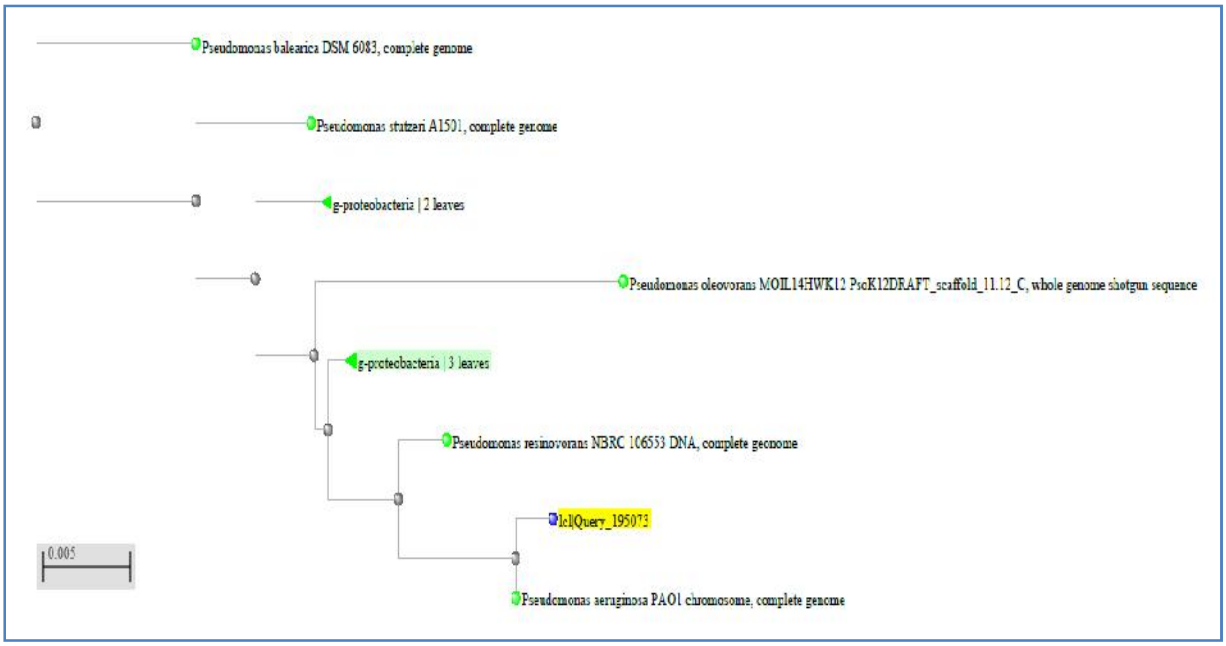

Fig. 3
Phylogenetic relationship of the isolate KMM8

Fig. 4
Phylogenetic relationship of the isolate KMM9

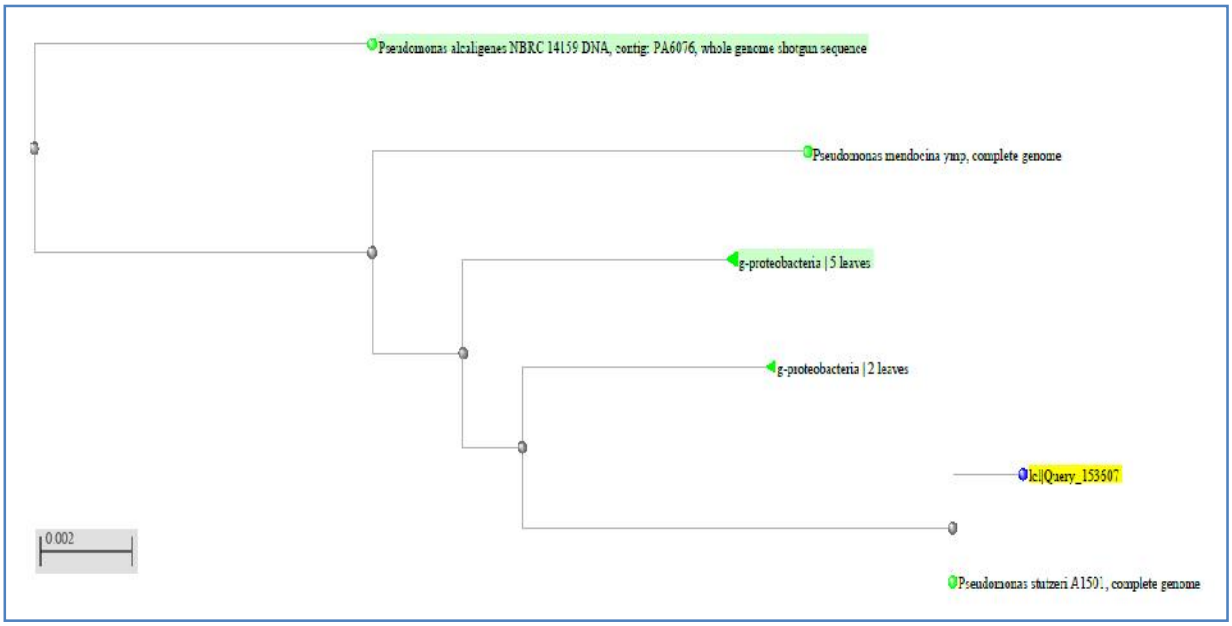

Fig. 5
Phylogenetic relationship of the isolate KMM10

The isolation and characterization of bacteria from coal samples first reported in 1908 and first time pure cultures of bacteria grown on brown coal samples was happened in 1910[29]. Since that time many researchers much focused on bacterial solubilization of different types of coal[30, 31]. Diversity of the bacterial communities in many media is extraordinary and high levels of bacterial diversity make quantifying and characterizing their communities a daunting task. However, few groups of aerobic bacteria utilize part of coal as growth substrate and metabolism in obligate conditions[32]. In the present investigation, we have isolated five bacterial strains that having the capability to degrade and utilize three PAHs namely, phenanthrene, anthracene and pyrene as sole carbon nutritional source. The isolation of bacteria from PAHs contaminated sites offers microorganisms with unusual properties and activities. The present study identified characteristics of isolated strains revealed the true diversity of microorganisms and their unique functionality which arise from their biological system that produce enzymes to make them tolerate or adapt to their environments[32]. The isolated bacterial strains of this study are *Bacillus cereus* KMM6 (KMM6), *Bacillus pseudomycoides* KMM7 (KMM7), *Pseudomonas aeruginosa* KMM8 (KMM8), *Bacillus cereus* KMM9 (KMM9) and *Pseudomonas stutzeri* KMM10 (KMM10). Many report published the occurrence of bacterial strains *Bacillus* sp. *Proteobacteria, Streptomyces badius, Streptomyces setoni, A. ferrooxidans* and *L. ferrooxidans* from coal mines[33,34]. The present results are in conformity with the many earlier findings[35,36].

CONCLUSIONS

The present study successfully isolated five novel distinct PAHs degrading bacteria at strains level and has been proposing high bacterial diversity in this coalmine site of Kothagudem, Khammam district of Telangana state, India. The present research is also focusing the significance of the isolation of PAHs degrading bacteria from PAHs innate compound stricture, coal which rarely considered for PAHs degrading bacterial isolations.

ACKNOWLEDGEMENTS

Author is very thankful to the Management of Singareni Collieries Company Limited, Kothagudem, Khammam District for their help for this research work. I am grateful to University Grants Commission for their financial support for this research via UGC-Minor Research Project.

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