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RESEARCH ARTICLE

EXISTENCE OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) DEGRADING BACTERIA IN COAL SAMPLES.

M. Poornachander Rao.

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Abstract

This study is conducted to isolate polycyclic aromatic hydrocarbons (PAHs) degrading bacteria from coal samples of an opencast coalmine. In this study five PAHs degrading bacteria were isolated on minimal salt medium (MSM) that priory enriched with a PAHs compound, phenanthrene. Lytic zone formations on MSM agar during culture growth also confirmed the PAHs degradation. Microscope assisted methods revealed the nature of cell type and Gram reaction type of the isolates as two are Gram-positive and three are Gram-negative and all are rod shaped bacteria. The isolates screened for their ability to grow on MSM broth that supplemented with three test PAHs compounds phenanthrene, anthracene or pyrene as sole source of carbon. In this assay, the isolates exhibited good range of growth on the tested three PAHs. Under morphological characterization and biochemical characterization the isolates recorded significant differentiation. Production of different enzymes on specific media determined as per standard protocols for the bacteria. Finally, the isolates characterized at molecular level by sequencing 16S rDNA genes. The isolated bacteria identified by the compilation of morphological, biochemical and molecular characterization data as *Bacillus thuringiensis* KMM1, *Achromobacter xylosoxidans* KMM2, *Bacillus cereus* KMM3, *Alcaligenes faecalis* KMM4 and *Brevibacillus laterosporus* KMM5.

Introduction:-

Coal has been become a more important energy source in the 21st century. It contains large quantities of organic and inorganic matter. When it burns chemical and physical changes take place and many toxic compounds are formed

Corresponding Author:- M. Poornachander Rao.

and emitted. PAHs are among those releasing compounds and are considered to pose potential health hazards because some PAHs are known carcinogens. Based on their toxicology, 16 PAHs are considered as priority pollutants by the USEPA (Liu 2008). PAHs of coal origin have getting great interest in recent environmental research since a huge state of coal mining for energy production in our nation and around the world.

Existence of wide variety of bacterial species in the nature is ubiquitous and has been reported from all environments sections. Bacterial species such as *Bacillus* sp. *Proteobacteria*, *Streptomyces badius*, *Streptomyces setoni*, *A. ferrooxidans* and *L. ferrooxidans* are known to be present in coal samples (Faison 1991; Laborda et al. 1997). Plenty of studies reported indigenous type of bacterial species from natural coal samples and their related products (Darland et al. 1970; Ledin and Pedersen 1996). However, presence of high density of bacteria is restrict for coal samples and till dated few bacterial strains reported in coal beds and their particles collected from coalmines (Williamson and Johnson 1990; Machulla 2007). Such type of bacteria can be isolated using MSM like media that supplemented with PAH compounds as sole carbon sources. The present study is aimed at the purification of enrichment cultures of bacteria obtained from coal samples of Manuguru opencast coal mines of Khammam district through conventional plating and characterization techniques (Jamal et al. 2016).

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 using minimal salt medium (MSM) was adopted from Dean-Ross et al. (2001). 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, one 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 bacterial isolates were purified by adopting the method of Kiyohara et al. (1982). 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 (John et al. 2012).

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. Method of biochemical characterization and their application in bacterial

taxonomy adopted from Cappuccino et al. (1996). The catalase test performed according to the method of Kubica and Pool, (1960). 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 using 16S rDNA gene sequences for PAHs degrading bacteria was adopted from Wang *et al* (1995). DNA extraction method was adopted from Gauthier *et al* (2003). 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:-

In the present study, five PAHs degrading bacteria were isolated from coal samples using minimal salt medium (MSM) broth enriched with 100 ppm concentration of phenanthrene (Table 1). After the isolation, they were purified by repeated streaking and sub-culturing on MSM agar and nutrient agar medium (sprayed with phenanthrene) for further confirmation of PAHs degradation and purification of the bacterial cultures (Table 1).

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

S. No.	Code of the Isolate	Characteristics
1	KMM1	Gram- positive, rod shaped
2	KMM2	Gram-negative, rod shaped
3	KMM3	Gram- positive, rod shaped
4	KMM4	Gram-negative, rod shaped
5	KMM5	Gram-negative, rod shaped

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

The PAHs degrading isolates then screened for their ability to grow on MSM enriched with phenanthrene, anthracene or pyrene at 100 ppm concentration as a sole carbon source and growth was recorded in terms of optical density (OD). The results are presented in Table 2. The isolates utilized phenanthrene, anthracene and pyrene for their growth and metabolism at higher levels and growth recordings are good. These results indicated the good abilities of bacterial isolatesfor these three tests PAHs compounds even as a source of carbon.

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	KMM1	0.03	0.04	0.02
3	KMM2	0.07	0.03	0.06
4	KMM3	0.04	0.04	0.05
5	KMM4	0.08	0.06	0.04
6	KMM5	0.06	0.07	0.06

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.

Table 3:- Morphological characterization of the PAHs degrading isolates.

S. No.	Code of the Isolate	Characteristics
1	**KMM1**	Colonies are spherical, opaque, have slightly raised elevation, regular margin and white to off-white in colour. Cells are motile and spore forming.
2	**KMM2**	Colonies are watery, translucent and convex, have regular and smooth margin and gray to white in colour. Isolate is motile (peritrichous) and non-spore forming.
3	**KMM3**	Colonies are small, irregular, flat with an undulate margin, rough and have dry texture and shiny light cream in colour. Cells are motile and sporulating.
4	**KMM4**	Colonies are small, spherical, have thin, spreading irregular edge and without pigmentation. Cells are motile (peritrichous) and non-spore forming.
5	**KMM5**	Colonies are round, smooth and white to yellowish in colour. Cells are motile (peritrichous) and spore forming.

Table 4:- Biochemical characterization of the bacterial isolates from coal samples.

S. No.	Test Performed	Reaction				
		KMM1	**KMM2**	**KMM3**	**KMM4**	**KMM5**
1	ONPG	Positive	Negative	Negative	Positive	Negative
2	Lysine Decarboxylase	Negative	Negative	Negative	Negative	Negative
3	Ornithine Utilization	Positive	Negative	Positive	Negative	Negative
4	Urease	Negative	Positive	Negative	Negative	Negative
5	Phenyl Alanin Deamination	Negative	Negative	Negative	Positive	Negative
6	Nitrate Reduction	Positive	Positive	Negative	Positive	Positive
7	H_2S Production	Negative	Positive	Negative	Negative	Negative
8	Citrate Utilization	Negative	Positive	Positive	Positive	Negative
9	Voges-Proskauer's Test	Negative	Negative	Positive	Negative	Negative
10	Methyl Red	Positive	Positive	Negative	Positive	Negative
11	Indole test	Negative	Negative	Positive	Negative	Positive
12	Malonate Utilization	Negative	Negative	Negative	Negative	Positive
13	Cytochrome Oxidase	Positive	Positive	Positive	Positive	Positive
14	Catalase	Positive	Positive	Positive	Positive	Positive
15	Lipase	Positive	Positive	Positive	Negative	Positive
16	Amylase	Positive	Positive	Positive	Negative	Negative

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 (Himedia KB003) under sterile conditions. The data produced during biochemical characterization presented in Table 4. In this study, the isolate KMM1 exhibited positive

reactions to the tests of ONPG, Ornithine utilization, nitrate reduction, methyl red, cytochrome oxidase, catalase, lipase and amylase. On the other hand, the isolate exhibited negative reactions to the tests lysine decarboxylase, urease, phenyl alanin deamination, H_2S production, citrate utilization, Voges-Proskauer's, Indole and malonate utilization. The isolate, KMM2 exhibited positive reactions to the tests of urease, nitrate reduction, H_2S production, citrate utilization, methyl red, cytochrome oxidase, catalase, lipase and amylase. On the other hand, the isolate exhibited negative reactions to the tests of ONPG, lysine decarboxylase, Ornithine utilization, phenyl alanin deamination, Voges-Proskauer's, Indole and malonate utilization. The isolate, KMM3 recorded positive reactions to the tests of Ornithine utilization, citrate utilization, Voges-Proskauer's, indole, cytochrome oxidase, catalase, lipase and amylase. On the other hand, the strain recorded negative reactions to the tests of ONPG, lysine decarboxylase, urease, phenyl alanin deamination, nitrate reduction, H_2S production, methyl red and malonate utilization. The isolate, KMM4 showed positive reactions to the tests of ONPG, phenyl alanin deamination, nitrate reduction, citrate utilization, methyl red, cytochrome oxidase and catalase. On the other hand, the same isolate showed negative reactions to the tests of lysine decarboxylase, Ornithine utilization, urease, H_2S production, Voges-Proskauer's, Indole, malonate utilization, lipase and amylase. The isolate, KMM5 exhibited positive reactions to the assays of nitrate reduction, Indole, malonate utilization, cytochrome oxidase, catalase and lipase. On the other hand, the isolate exhibited negative reactions to the assays of ONPG, lysine decarboxylase, Ornithine utilization, urease, phenyl alanin deamination, H_2S production, citrate utilization, Voges-Proskauer's, methyl red and amylase.

Molecular characterization of the pahs degrading isolates:-

Characterization of the PAHs degrading isolates at molecular level was studied based upon their respective 16S rDNA sequences and these sequences were analyzed by using BLAST that available at NCBI site followed by comparison with other available reference nucleotide sequences that deposited in NCBI and EMBL databases. In this study, the strain KMM1 recorded the maximum identity (99%) and query cover (100%) with seven *Bacillus thuringiensis* strains on NCBI blast analysis. Phylogenetic tree for the isolate constructed using significant gene similarities alignments showing bacterial strains (Fig. 1). The results revealed the unique and unmerge characteristics of the isolate with any other bacterial strain. Hence, we named this isolate as *Bacillus thuringiensis* KMM1 and got its NCBI accession number, KX371870. The isolate, KMM2 showed the maximum identity (99%) and query cover (100%) with many *Achromobacter xylosoxidans* strains on NCBI blast analysis. Phylogenetic tree for this strain was constructed using significant gene similarities alignments showing bacterial strains (Fig. 2). The results revealed that the isolate is unique with unmerge characteristics with any other bacterial strain. Hence, we named it as *Achromobacter xylosoxidans* KMM2 and its NCBI accession number is KX036663. The isolate, KMM3 exhibited the maximum identity (99%) and query cover of 100% with many *Bacillus cereus* strains on blast analysis. Phylogenetic tree for the isolate was constructed based on their highest gene sequence similarities with other strains (Fig. 3). The results revealed that the isolate is unique and new with unmerge characteristics with any other bacterial strain. Therefore, we have given name to this isolate as *Bacillus cereus* KMM3 and its NCBI accession number is KX371872. The strain, KMM4 showed the maximum identity (99%) and query cover (100%) with *Alcaligenes faecalis* strain DSM complete genome (Sequence ID: NZ AUB0100026.1). Phylogenetic tree for the isolate was constructed using significant gene similarities alignments showing bacterial strains (Fig. 4). The present results indicated novelty of the isolate at strain level. Hence, we named this strain as *Alcaligenes faecalis* KMM4 and its NCBI accession number is KX149020. The isolate, KMM5 recorded the maximum identity (99%) and query cover of 100% with *Brevibacillus laterosporus* LMG strain complete genome (Sequence ID: NZ CP007806.1). Phylogenetic tree for the isolate constructed using significant gene similarities alignments showing bacterial strains (Fig. 5). The results revealed that the isolate is unique with unmerge characteristics with any other bacterial strain. Hence, named it as *Brevibacillus laterosporus* KMM5 and its NCBI accession number is KX149021.

Fig 1:- Phylogenetic relationship of the isolate KMM1.

Fig 2:- Phylogenetic relationship of the isolate KMM2.

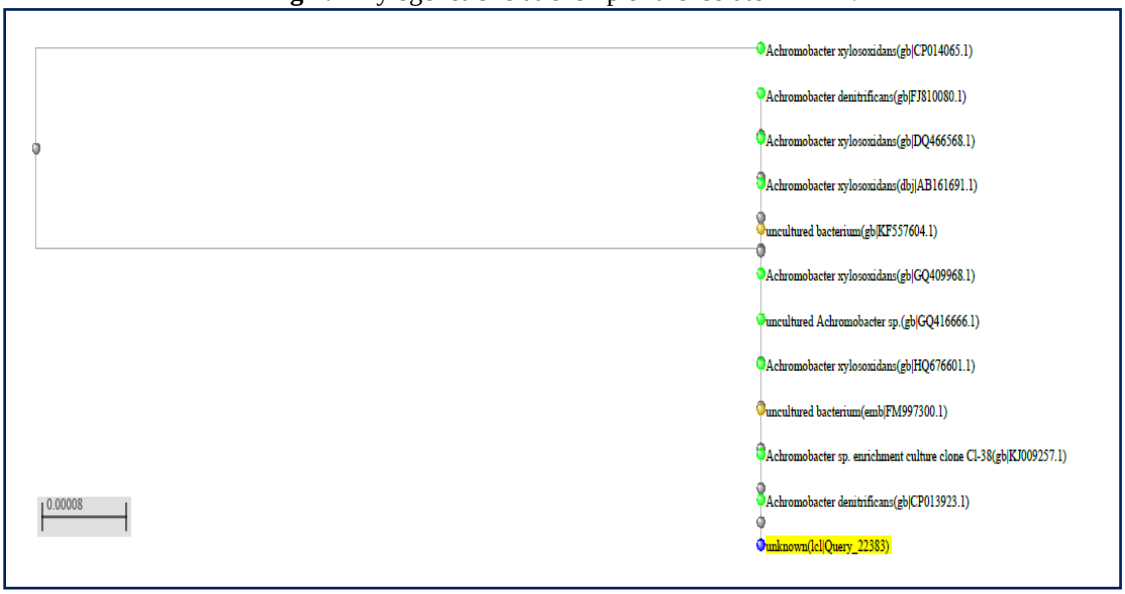


Fig 3:- Phylogenetic relationship of the isolate KMM3.

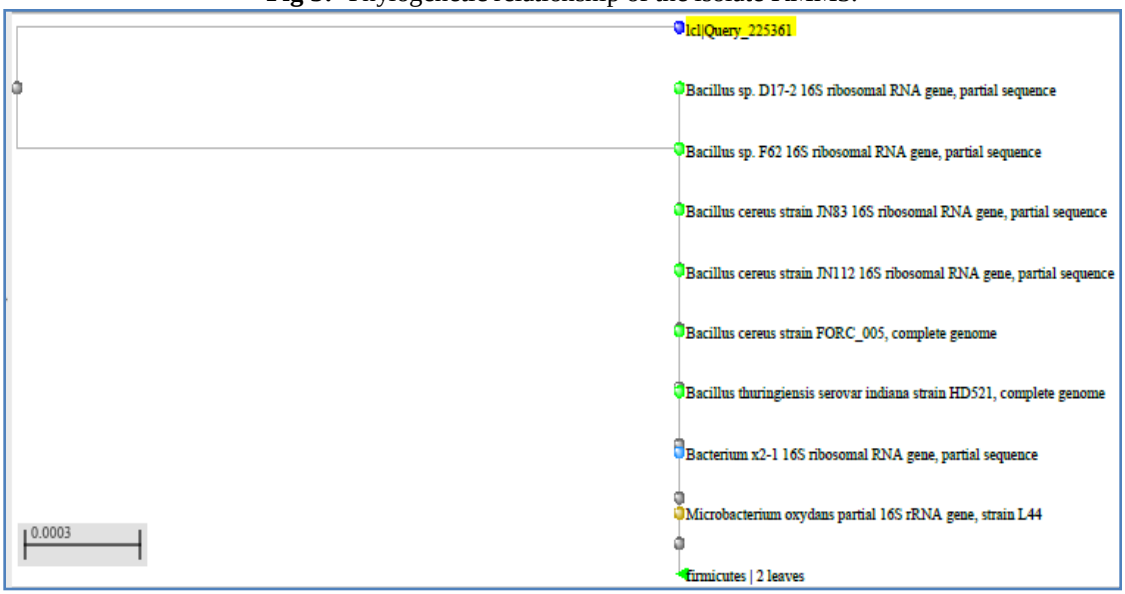


Fig 4:- Phylogenetic relationship of the isolate KMM4.

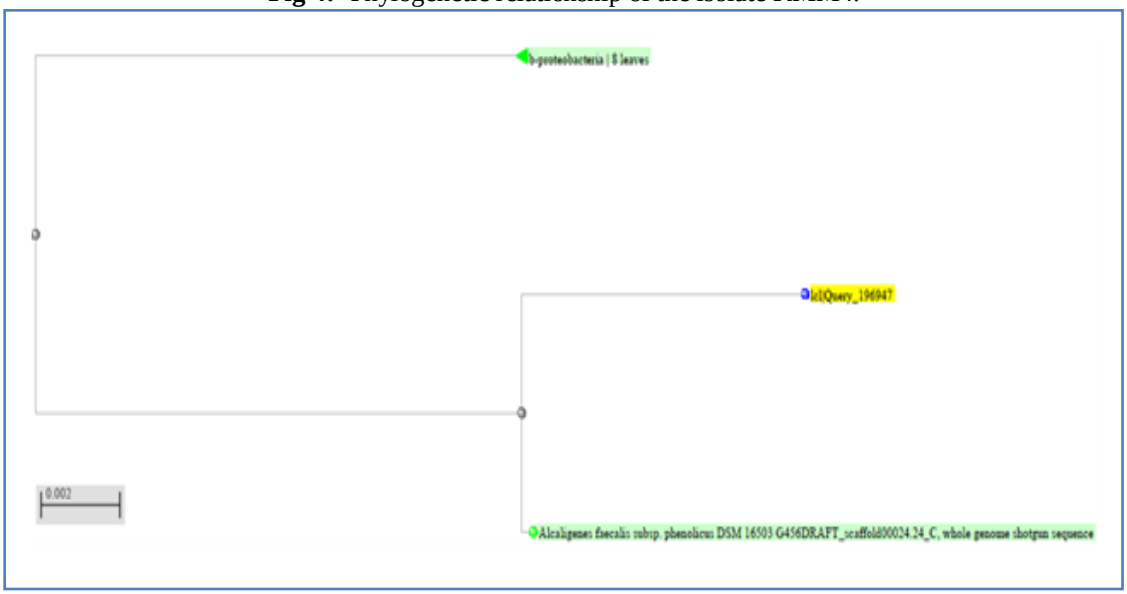
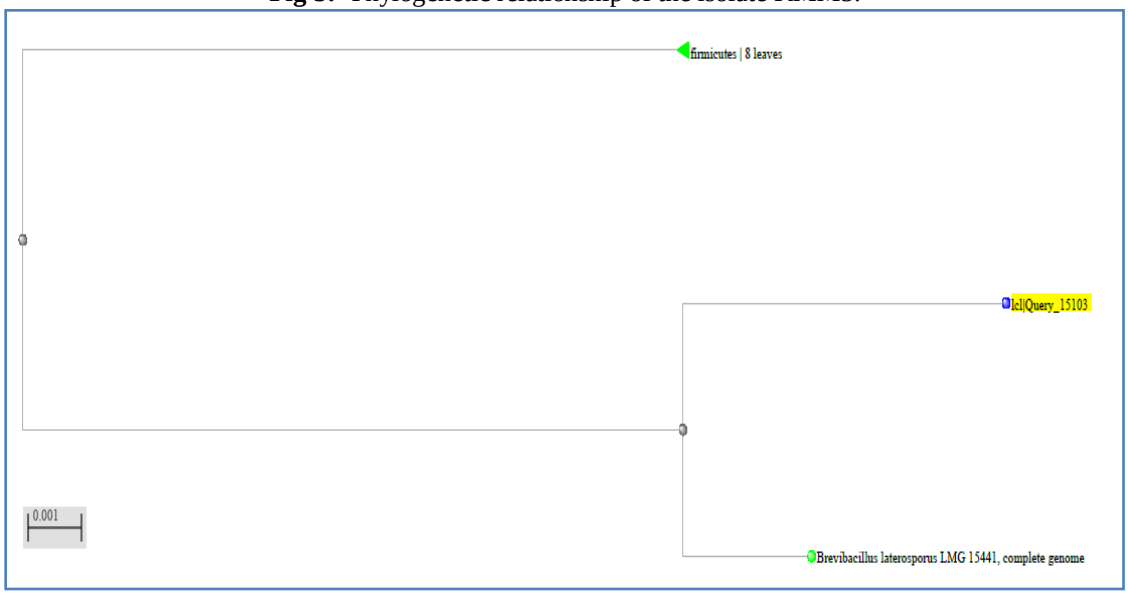


Fig 5:- Phylogenetic relationship of the isolate KMM5.



Discussion:-

Polycyclic aromatic hydrocarbons are heady environmental pollutants that comprise fused aromatic rings. They are found almost everywhere in the environment and are typically formed during unfinished burning of organic materials such as wood, coal, oil, gasoline etc. Natural crude oil and coal deposits contain significant amount of PAHs arising from chemical conversion of natural products molecules such as steroids (Roy 1995; Fetzer 2000; Ukiwe 2013). Biodegradation is one of the forms of bioremediation applied to treat soils, water or sediments contaminated with PAHs. It is the use of microorganisms to degrade or detoxify many environmental pollutants (Bamforth & Singleton 2005). Biodegradation is also a clean-up method that presents the possibility to eliminate organic contaminants with the aid of natural biological activity available in the substrate (Zeyaullah et al 2009). This method also optimized by involving many factors among which are the existence of a microbial consortia capable of degrading the pollutant, the bioavailability of the contaminant to microbial attack and certain environmental factors (soil type, temperature, soil pH, oxygen level of soil, electron acceptor agents, nutrient content of soil) contributing to microbial growth (Gratia et al 2006). Hence, identification of bacterial populations that having the ability to degrade PAHs compounds from coal samples is prerequisite and this aim is undertaken in the present study. Five efficiently PAHs degrading bacteria isolated from coal samples of Manuguru opencast coalmines on MSM priory enriched with phenanthrene and observing lytic zone formations followed by isolates purification. Among the five isolates three are Gram-positive and two are Gram-negative bacteria and all are rod shaped. Marsh and Norris (1983) and Sethy and Behera (2009) also published similar results.

On the screening for PAHs degradation ability using phenanthrene, anthracene and pyrene provided as sole source of carbon the isolates recorded good readings of grow and indicated high potential of PAHs degradation. These results coincide with the findings of Bogan *et al* (2003) and Bisht *et al* (2010). Hence, all the isolates then selected for the characterization, identification and nomenclature and these stages proceeded with three successive stages of morphological characterization, biochemical characterization and molecular characterization followed by identification and nomenclature.

Morphologically all the isolates are characteristically different at both culture and cell types. The biochemical properties dependant characterization carried out by 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 the biochemical characterized media. Finally the isolates are characterized at molecular level using 16S rDNA sequences (Lane *et al* 1985). 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 upon gene similarity percentages of the isolates (16S rDNA gene

sequences with other bacterial sequences) phylogenetic trees constructed using Neighbor-joining method and taxonomical positions of the isolates were determined.

The bacterial population that exists on coal and their products utilize constituents of coal as growth substrates for their metabolism under obligate conditions (Galle, 1910). As many researchers proposed the main mechanism applied in this is solubilization (Fakoussa and Hofrichter 1999; Laborda et al. 1997). In the present investigation we have isolated only five bacterial strains that possessing capability to degrade PAHs as sole carbon nutritional source. These results indicated poor existence of bacterial diversities on fresh coal samples of coalmines.

Relatively few microbiologists seriously considered coal samples for the studies of ability to modify the physicochemical structure of coal and PAHs degrading bacterial population. This might be due to they prefer simple sugars, organic acids and the like as substrates for microbial activity, and they try to avoid the use of too complex substrates such as coal. However in this study we successfully isolated five bacterial strains are *Bacillus thuringiensis* KMM1 (KMM1), *Achromobacter xylosoxidans* KMM2 (KMM2), *Bacillus cereus* KMM3 (KMM3), *Alcaligenes faecalis* KMM4 (KMM4) and *Brevibacillus laterosporus* KMM5 (KMM5) and many report published the presence of bacterial strains like *Bacillus* sp. *Proteobacteria, Streptomyces badius, Streptomyces setoni, A. ferrooxidans* and *L. ferrooxidans* from coalmines (Faison 1991; Laborda et al. 1997). A recent studies of Ramesh et al. (2014) and Jamal (2016) much focused the essentiality of the coal samples for the isolations of diversified bacterial species. The present study successfully isolated five novel PAHs degrading bacteria at strains level and has been proposing high level bacterial diversity in this coalmine site of Manuguru, 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.

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